UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
INSTITUTO DE BIOCIÊNCIAS – RIO CLARO

PROGRAMA DE PÓS-GRADUAÇÃO EM ZOOLOGIA

VARIAÇÕES GENÉTICAS E MORFOMÉTRICAS ENTRE AS ARANHAS *PTERINOPELMA LONGISTERNALIS* (BERTANI 2001) E *PTERINOPELMA ROSEUS* (MELLO-LEITÃO, 1923) (MYGALOMORPHAE, THERAPHOSIDAE)

WOLF J MOELLER

Rio Claro – SP
2023

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Orientador: Dr. José Paulo Leite Guadanucci

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TÍTULO DA DISSERTAÇÃO: ANÁLISE FILOGEOGRÁFICA DAS ARANHAS *VITALIUS LONGISTERNALIS* BERTANI 2001 E *VITALIUS ROSEUS* (MELLO-LEITÃO, 1923) (MYGALOMORPHAE, THERAPHOSIDAE)

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RESUMO

Aranhas da infraordem Mygalomorphae, conhecidas como caranguejeiras, se caracterizam pela alta homogeneidade morfológica e hábitos de vida sedentários, que refletem em baixa vagilidade e capacidade de dispersão. Estas características normalmente ocasionam forte estruturação na genética populacional. Desta forma, tornam-se excelentes organismos modelos em estudos integrativos pautados pela filogeografia e a morfometria geométrica. As espécies irmãs *Pterinopelma longisternalis* e *Pterinopelma roseus* (Theraphosidae), possuem ampla distribuição na região sul do Brasil, com possível co-ocorrência no oeste do Estado de Santa Catarina. Dada suas relações filogenéticas e suas semelhanças morfológicas, é proposta uma análise integrativa usando métodos morfométricos, para testar se existem diferenças morfológicas, e genéticas, para recuperar a história evolutiva, entre as duas espécies. Os resultados das análises morfométricas conseguiram diferenciar com sucesso *P. longisternalis* de *P. roseus*, pela forma do esterno (apesar do aspecto contínuo desse caráter) e pela morfologia dos bulbos copulatórios dos machos. Além disso, foi proposta uma análise genética para explorar as possíveis variações intra e interespecíficas das duas espécies. Os resultados moleculares apontaram a existência de uma terceira linhagem genética, com morfologia críptica a *P. longisternalis.*

Palavras chaves: Theraphosidae, Mygalomorphae, Morfometria, Sistemática Filogenética

ABSTRACT

Mygalomorphae spiders compound an infraorder, known as tarantula. They are characterized by high morphological homogeneity and sedentary lifestyle habits, which reflect in low vagility and dispersal capacity. These characteristics normally cause strong structuring in population genetics. In this way, they become excellent model organisms in integrative studies guided by phylogeography and geometric morphometry. The sister species *Pterinopelma longisternalis* and *Pterinopelma roseus* (Theraphosidae), have a wide distribution in southern Brazil, with possible co-occurrence in western Santa Catarina State. Given their phylogenetic relationships and their morphological similarities, an integrative analysis is proposed using morphometric methods, to test whether there are morphological and genetic differences, to recover the evolutionary history, between the two species. The results of the morphometric analyzes were able to successfully differentiate *P. longisternalis* from *P. roseus*, by the shape of the sternum (despite the continuous appearance of this character) and by the morphology of the male copulatory bulbs. In addition, a genetic analysis was proposed to explore the possible intra and interspecific variations of the two species. Molecular results pointed to the existence of a third genetic lineage, with cryptic morphology to *P. longisternalis*.

Keywords: Theraphosidae, Mygalomorphae, Morphometry, Phylogenetic Systematics

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INTRODUÇÃO

As aranhas compõem a ordem Araneae sendo este o quinto táxon animal mais diversificado, representado por mais de 50 mil espécies descritas e com distribuição global (WORLD SPIDER CATALOG, 2023; ZHANG, 2013). As principais sinapomorfias do grupo são a produção de seda por meio de órgãos especializados, denominados fiandeiras, e a presença de um bulbo copulatório nos tarsos dos pedipalpos dos machos, uma estrutura especializada para a transferência de esperma (WHEELER *et al.*, 2017). Possuem hábito predatório e apresentam estratégias de forrageamento diversificadas entre os diferentes grupos (FOELIX, 2011).

Segundo a hipótese filogenética de Platnick & Gertsch (1976), a ordem Araneae é subdividida em duas subordens: Mesothelae, representada pela infraordem Liphistiomorphae, com distribuição exclusiva na região Indo-Malaia e caracterizadas por reterem caracteres considerados primitivos, como segmentação externa do abdômen, dois pares de pulmões foliáceos e quatro pares de fiandeiras dispostas na face ventral, inseridas medialmente no abdômen; Opisthothelae, com ampla distribuição mundial e caracterizadas pela posição posterior das fiandeiras no abdômen, representada pelas infraordens Mygalomorphae e Araneomorphae (FOELIX, 2011).

As aranhas da infraordem Mygalomorphae são popularmente conhecidas como caranguejeiras, tarântulas, aranhas teia de funil ou aranhas de alçapão. O grupo é composto por cerca de 3.000 espécies, distribuídas em 30 famílias, representando cerca de 6% da diversidade total das aranhas (OPATOVA *et al.,* 2019; MONTES DE OCA *et al.*, 2022; WORLD SPIDER CATALOG, 2023). De modo geral, a morfologia das aranhas Mygalomorphae é conservada, marcada pela retenção de caracteres plesiomórficos em relação à Araneomorphae (OPATOVA *et al.,* 2019). Ainda, alguns dos caracteres morfológicos do grupo apresentam pouca variação, especialmente entre espécies próximas, o que pode dificultar a sua codificação (RAVEN 1985, BERTANI 2000). Por isso, é comum que em resultados filogenéticos, ocorram recuperações homoplástica de alguns estados, podendo repercutir em relacionamentos com suportes baixos e/ou divergentes (RAVEN 1985; GOLOBOFF, 1993; BOND & OPELL, 2002; PÉREZ-MILES & PERAFÁN, 2017).

A codificação de caracteres genéticos pode ser uma alternativa ou um complemento ao uso dos caracteres morfológicos em análises filogenéticas. As hipóteses de relacionamento moleculares se baseiam na variação genética e no acúmulo de mutações. Com os avanços nas tecnologias de biologia molecular, bioinformática e o subsequente barateamento dessas técnicas, aumentaram a popularização desses métodos (HEDIN & BOND, 2006; BOND *et al.*, 2012; HEDIN *et al.*, 2018, OPATOVA *et al.,* 2019, MONTES DE OCA *et al.*, 2022).

A família Theraphosidae é a mais diversificada das aranhas Mygalomorphae, com 1055 espécies descritas distribuídas em 13 subfamílias encontradas principalmente em regiões tropicais e subtropicais (WORLD SPIDER CATALOG, 2023). A subfamília Theraphosinae é a mais diversa de Theraphosidae com espécies distribuídas pelas zonas temperadas e tropicais das Américas. A característica mais notável desta subfamília é a presença de diferentes tipos de cerdas urticantes no opisthosoma (BERTANI, 2000; BERTANI & GUADANUCCI, 2013).

O gênero *Vitalius* é um dos gêneros mais representativos da subfamília Theraphosinae no Brasil, distribuído por toda a região sul e Sudeste do país. Foi originalmente proposto por Lucas, Silva & Bertani (1993) durante a revisão do gênero *Pamphobeteus*, e posteriormente revisado por Bertani (2001), que propôs a monofilia do grupo pautada nos seguintes caracteres taxonômicos: êmbolo com uma quilha subapical triangular nos machos, ausência de cerdas estridulatórias, espermateca com dois receptáculos separados por uma base fortemente esclerotizada, e presença de cerdas urticantes tipo III nas fêmeas. Em contraste ao relacionamento cladístico proposto por Bertani (2001), a monofilia do gênero *Vitalius* não é suportada nas análises filogenômicas (UCEs) de Galleti-Lima *et al.* (2023). Os dados filogenômicos apontam que as espécies *Vitalius longisternalis* e *Vitalius roseus* são irmãs e formam um clado a parte com *Pterinopelma vitiosum* (Keyserling, 1891), e sugere a transferências destas espécies de *Vitalius* para *Pterinopelma.* Além disso, os resultados deste trabalho recuperam *Pterinopelma* como grupo irmão de *Vitalius,* sendo estes gêneros diferenciados apenas por caracteres morfológicos sexuais sutis.

Dentre as espécies de *Pterinopelma*, a espécie *P. vitiosum* é a que possui os caracteres morfológicos mais destoantes, apresentando esterno mais largo do que longo e quilha sub-apical reduzida e não pronunciada nos machos (BERTANI *et al.,* 2011, fig. 1-5). Para *Pterinopelma longisternalis* o único caráter diagnóstico citado é "esterno muito mais longo do que largo" (BERTANI, 2001), característica que também é observada em alguns espécimes de *Pterinopelma roseus*. Além disso, o aspecto geral dos bulbos copulatórios de *P. longisternalis* e *P. roseus* é parecido, ambas as espécies possuem quilha sub-apical pronunciada, dificultando a diferenciação por este caráter. Nas fêmeas, a ausência de caracteres diagnósticos é a mais evidente devido a alta semelhança do formato das espermatecas.

Uma forma de discriminar esses caracteres morfológicos é através de técnicas de morfometria geométrica. Tais métodos visam recuperar a forma geométrica das estruturas morfológicas através de coordenadas obtidas por pontos anatômicos homólogos (*landmarks*) (WEBSTER *et al.*, 2010). Com base nessas coordenadas, é possível recuperar a forma e testar a diferenciação por meio de análises multivariadas com suporte estatístico (ROHLF & MARCUS, 1993). Em estruturas morfológicas onde pontos anatômicos homólogos não exibem fácil localização (como bulbos e espermatecas), uma alternativa viável é o uso de análises por contornos fechados (ROHLF, 1990). Um dos principais métodos é a análise elíptica de Fourier, que tem sido constantemente abordada em estruturas de contornos complexos, como de crustáceos e insetos (KUHL & GIARDINA, 1982; ROHLF & ARCHIE, 1984; FERSON *et al.*1985; TORT, 2003; ZAHN & WANG, 2012; WEN *et al.*2015). Este método recupera informações da forma da estrutura, independentemente do tamanho, posição e rotação do contorno, utilizando os coeficientes de harmônicos (CRAMPTON, 1995).

Além da similaridade morfológica entre *P. longisternalis* e *P. roseus*, as duas espécies apresentam distribuição aparentemente parapátrica, com uma estreita area em simpatria. *P. longisternalis* se distribui pelos estados do Paraná e Santa Catarina, majoritariamente em fragmentos de Floresta Ombrófila Mista, enquanto *P. roseus* se distribui desde o centro-oeste do Rio Grande do Sul até o Oeste de Santa Catarina. Ambas as espécies compartilham distribuição em regiões de contato entre a Floresta Ombrófila Mista e a Floresta Estacional Semidecidual. O relevo dessas áreas é marcado pela presença de descontinuidades geológicas, como serras e escarpas e rios de grande porte como os rios Iguaçu e Uruguai. (VITTE & GUERRA, 2004).

Não se sabe qual a influência dessas características geográficas sobre a diversidade populacional e a distribuição de ambas as espécies. A influência de barreiras biogeográficas na delimitação de espécies é um dos temas estudados pela filogeografia (TURCHETTO & ZOLET *et al.,* 2013). Proposto por Avise *et al.* (1987), os métodos filogeográficos utilizam as variações no DNA mitocondrial para recuperar os relacionamentos filogenéticos. Além disso, esses dados podem ser utilizados para unir resultados de processos macroevolutivos, como a sistemática filogenética e a geografia histórica, a processos microevolutivos, como a genética de populações (AVISE *et al.*, 1987; BEHEREGARAY, 2008; AVISE, 2009).

A diferenciação morfológica entre *P. longisternalis* e *P. roseus* ainda não é clara, devido aos seus caracteres diagnósticos apresentarem estados similares. Também não são conhecidas variações morfológicas e/ou genéticas entre as populações de ambas as espécies. Diante disso, é apresentada uma investigação com base em análises integrativas, que unem métodos morfométricos para testar a existência de variações morfológicas com métodos genéticos para testar a existência de linhagens populacionais e o seu histórico de diversificação. Essas análises propõem um método menos especulativo sobre a evolução do grupo, além de contribuir no conhecimento de processos de diferenciação morfológica e genética de aranhas Theraphosidae.

A presente dissertação está coma sua discussão dividida em dois capítulos. O primeiro aborda os resultados morfométricos obtidos por meio de análises de de morfometria geométrica e análises de contorno. O segundo capítulo apresenta os resultados preliminares das análises filogenéticas e discute as topologias recuperadas.

MATERIAIS E MÉTODOS

Coleções analisadas

Foram examinados e utilizados matérial das seguintes coleções:

CAD, Coleção Aracnológica Diamantina, Rio Claro, Universidade Estadual Paulista Júlio de Mesquita Filho – *campus* Rio Claro, Rio Claro, São Paulo, Brasil – J.P.L. Guadanucci.

IBSP, Coleção Aracnológica do Instituto Butantan, São Paulo, Brasil – A. D. Brescovit;

MCTP, Museu de Ciências e Tecnologia da PUCRS, Porto Alegre, Rio Grande do Sul, Brasil **-** R. A. Teixeira;

MHNCI, Museu de História Natural Capão da Imbuia, Curitiba, Paraná, Brasil – A. Silva;

MZSP, Museu de Zoologia da Universidade de São Paulo, São Paulo, Brasil – R. Pinto da Rocha.

Obtenção e preservação de espécimes

Foram realizadas três expedições de campo que amostraram dez localidades (Tabela 1) para obtenção de material fresco. Os espécimes foram coletados de forma ativa (através de técnicas que consistem em revirar rochas, troncos e folhiço, investigar fendas e escavar tocas). A primeira expedição foi realizada em dezembro de 2020 (localidades 3 e 8), a segunda em maio de 2021 (1, 2, 4, 5 e 6), e a terceira em Janeiro de 2022 (4 e 10). Todo o material coleto foi fixado e depositado no acervo da Coleção Aracnológica Diamantina (CAD). O material analisado e utilizado da espécie *P. roseus* foram doados da coleção particular do Msc. Leandro Malta Borges (LMB) para o CAD.

Tabela 1. Localidades das expedições de coleta.

Ponto	Localidade	Município	Estado	País
1	FLONA Piraí do Sul	Piraí do Sul	Paraná	Brasil
2	FLONA Assunguí	Campo Largo	Paraná	Brasil
3	FLONA Irati	Irati	Paraná	Brasil
4	FLONA Caçador	Caçador	Santa Catarina	Brasil
5	FLONA Chapecó	Chapecó	Santa Catarina	Brasil
6	FLONA Três Barras	Três Barras	Santa Catarina	Brasil
7	PARNA Parque das Araucárias	Passos Maia	Santa Catarina	Brasil
8	APA Municipal de Pitanga	Pitanga	Paraná	Brasil
9	APA Municipal de Palmas	Palmas	Paraná	Brasil
10	REVIS Campos de Palmas	Palmas	Paraná	Brasil

Definição dos terminais utilizados

Para a construção das árvores filogenéticas, foram utilizados espécimes de *P. longisternalis* e *P. roseus*, coletadas ao longo do polígono de ocorrência e distribuição das espécies. Como grupo externo, foram utilizados exemplares dos gêneros *Calisoga sp.*

Simon, 1889 - Nemesiidae*,* *Synothele* sp. Simon, 1908 – Barychelidae *e Nhandu tripepii* (Dresco, 1984) Theraphosidae cuja sequencias foram extraídas do GenBank (NCBI).

Análises morfométricas

Para investigar as variações morfológicas entre *P. longisternalis* e *P. roseus*, foram realizadas análises morfometria dos caracteres taxonômicos utilizados nas diagnoses como esterno, bulbo copulador dos machos (vista prolateral, excluindo o subtegulum) e espermateca das fêmeas. Para isto, foram obtidas imagens através de uma câmera fotográfica digital (Leica MC170HD) acoplada a um estereomicroscópio (Leica M205C). Para a investigação das variações dos esternos, foram conduzidas análises de morfometria geométrica nas estruturas. Para isso, foi utilizado o *software* TPSDIG32 *P.* 1.31 para inserção de coordenadas de marcos anatômicos (*landmarks*) nas fotografias obtidas, e esses dados submetidos no *software* MorphoJ *P.*1.05 para conduzir as análises dos componentes principais, correlação canônica e análises discriminantes (ROHLF, 2001; KLINGENBERG, 2011). Para as estruturas reprodutivas, optou-se pelo uso de análises de elípticos de Fourier, devido a dificuldade em estabelecer pontos de ancoragem homólogos nestas estruturas. Antes das análises, as imagens obtidas foram tratadas no software Photoshop 20.0.0 (CS Photoshop, 2020), onde tiveram o fundo removido e o interior das estruturas escurecidos. A análise foi conduzida no software R Statistics utilizando os pacotes: Geomorph (BONHOMME *et al.*, 2014), Morpho (SCHLAGER, 2017) e Mommocs (ADAMS *et al.*, 2021).

Extração, sequenciamento e amplificação de DNA

O material genético foi extraído da musculatura do fêmur da perna III direita das aranhas, utilizando o kit de extração GenElute^{TM} (Sigma-Aldrich), seguindo instruções do fabricante.

Para as análises moleculares foi utilizado o gene mitocondrial citocromo oxidase I (COI) amplamente utilizado em análises filogenéticas (FOLMER *et al.*, 1994). Os primers utilizados para o COI são: LCO1490 (FOLMER *et al.*, 1994) e C1-N-2776 (HEDIN & MADDISON, 2001) com as adaptações propostas por Hamilton *et al.* (2011) (Tabela 2).

Tabela 2. Genes e Primers utilizados nas análises filogenéticas de *Pterinopelma longisternalis* e *Pterinopelma roseus*.

Primer	Reference	Sequency	Direction
LCO1490	Folmer *et al.* (1994)	5'-GGTCAACAAATCATAAAGATATTGG-3'	foward
LCO1490aphonopelma	Hamilton *et al.* (2011)	5'-TTTCTACTAATCACAAGGATATYGG-3'	foward
LCO1490cg	Hamilton *et al.* (2011)	5'-TTTCTACTAATCACAAGGATATTGG-3'	foward
LCO1490chalcodes	Hamilton *et al.* (2011)	5'-TTTCCACAAACCACAGGGATATCGG-3'	foward
LCO1490reversum	Hamilton *et al.* (2011)	5'-TTTCTACTAATCATAGGGATATTGG-3'	foward
C1-N-2776	Hedin & Maddison (2001)	5'-GGATAATCAGAATATCGTCGAGG-3'	reverse

O processo de amplificação dos genes seguiu o método de DNA-PCR de Sanger. As amostras foram quantificadas previamente com uso de um espectrofotômetro NanoDrop ND-1000 da Thermo Fisher Scientific. Para as soluções, utilizou-se o kit de amplificação Platinum™ Taq DNA Polymerase da Thermo Fisher Scientific seguindo as instruções do fabricante com exceção dos ciclos do termociclador na qual foi utilizado o protocolo proposto por Hamilton *et al.*2014. A verificação do resultado da PCR foi obtida com a observação do gel de agarose 1% sobre luz UV após eletroforese, com amostras evidenciadas por GelRed^{TM}. O tamanho do fragmento amplificado foi estimado por meio de comparação com amostra de padrão de peso molecular (DNA *ladder*). Os produtos da PCR foram novamente quantificados e diluídos à 50 ng/ul em água ultrapura, purificados com uso de ExoSAP-IT da Thermo Fisher Scientific e as amostras enviadas para a Macrogen Korea para sequenciamento.

DESENVOLVIMENTO

Capítulo 1

Morphometric delimitation of *Pterinopelma longisternalis* (Bertani, 2001) and *Pterinopelma roseus* (Mello-Leitão, 1923).

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Abstract: Delimiting morphological characters for phylogenetic studies in Mygalomorphae taxa is a challenge. The group has a reduced number of these sinapomorphic characters, which are generally conservative. This high incidence of homogeneous characters leads to the use of continuous characters in the species diagnosis. The Theraphosidae species *P. longisternalis* and *P. roseus* are considered sister species. The main diagnostic characters that distinguish those species are the sternum length, and the genitalia morphology. However, these characters show variations that can make difficult to discriminate these two species. We test the significance of these characters in recovering these two species using two methods of morphogeometric analyzes: the Landmark-based analysis to explore the sternum morphometric variations and Fourier ellipses analyzes to explore the shape variations on males palpal bulbs and females spermathecae. Our results showed that the sternum length and the embolus width of *P. longisternalis* and *P. roseus* are statistically different and, thus, are reliable characters to diagnose the species. The same was not true for the female spermatheca, suggesting that is a week character for comparing those species.

Keywords: Geometric morphometrics, Elliptical Fourier, Mygalomorphae.

INTRODUCTION

Theraphosidae is the most diverse family of Mygalomorphae spiders, with thirteen subfamilies mostly found in tropical and subtropical regions (World Spider Catalog 2023). Five of these subfamilies have representatives in South America: Aviculariinae, 'Ischnocolinae', Psalmopoeinae, Schismatothelinae, and Theraphosinae. (Guadanucci 2014; Lüddecke *et al.* 2018). Theraphosinae is the most diverse of all Theraphosidae subfamilies, distributed across New World temperate and tropical zones. The most notable character of this subfamily is the presence of different types of urticating setae on the dorsum of the abdomen. (Bertani 2000; Bertani & Guadanucci, 2013).

In general, Mygalomorph spider shows a high incidence of homogeneous characters, which are generally conservative, causing more homoplastic reconstructions when compared to Araneomorphae (Bond & Opell, 2002; Pérez-Miles & Perafán, 2017). These characteristics may lead the use of continuous characters in the taxonomical species diagnosis. An example of this is in the theraphosid genus *Pterinopelma* Pocock, 1901. This genus is actually composed by the species *Pterinoplema vitiosum* (Keyserling, 1891), *Pterinopelma longisternalis* (Bertani, 2001) and *Pterinopelma roseus* (Mello-Leitão, 1923). The monophyly of the group was proposed by Galleti-Lima *et al.* (2023) which, through the results of an phylogenomic analysis, transferred the species *P. longisternalis* and *P. roseus* from *Vitalius* Lucas, Silva & Bertani, 1993. However, despite the authors proposing an updated diagnosis for the genus *Pterinopelma*, the species diagnoses remain the same proposed by Bertani (2001) for *P. longisternalis* and *P. roseus* and Bertani, Nagahama & Fukushima (2011) for *P. vitiosum.*

If compared the main morphological characters of these three species, *P. vitiosum* can be distinguished from the others by the sternum wider than long and by the males palpal bulb with a sub-apical keel small and flattened. In other hand, the species *P. roseus* and *P. longisternalis* are more difficult to be distinguish from each other. The only synapomorphy proposed to *P. longisternalis* is "sternum much longer than wide" (Bertani, 2001). However, it is noticed that some specimens of *P. roseus* may have the sternum longer than wide too. How longer than wide is the sternum of *P. longisternalis* make its diagnosis unclear, which can lead to misidentification the species. Furthermore, the other diagnostical character between these two species is the shape variation of males and females reproductive structures. In males, the palpal bulb show discrete variations in embolous length, and both species have presence of a prominent subapical keel. In females, the spermatheca shape is almost equal, not being possible to distinguish between species. This set of similarities makes it difficult to distinguish *P. longisternalis* from *P. roseus*.

The three species show close geographical distribution, occurring through the Southern region of Brazil (Figure 1). The most distribution records of *P. longisternalis* are in Mixed Ombrophylous Forest areas of Paraná and Santa Catarina states. The species records for *P. roseus* show its distribution from the Deciduous Forest areas of the western region of Santa Catarina states to grassland areas in the Rio Grande do Sul state. (Figure 1). Finally, *P. vitiosum* has a known occurrence in the northern region of the state of Rio Grande do Sul, in regions of Subcaducifolious Atlantic Forest (Bertani *et al.*, 2012).

These areas have the presence of geological discontinuities, such mountains, cliffs and large rivers such as the Iguaçu and Uruguay rivers (Vitte & Guerra, 2004). The influence of these geographic characteristics on the population diversity and morphological variations is unknown. However, such characteristics may have influence on the distribution of population lineages and on speciation processes (Avise *et al.*, 1987).

To test de specie recovery by morphological characters, we applied a combined morphogeometric. The Landmark-based method is used to analyse the sternum structure which presents clear anatomical homologous points (landmarks). For male palpal bulbs and female spermatheca, these points are not clear, so the morphometric variation are be compared by Elliptic Fourier Analysis (EFA). This method uses coordinates or closed contour to recover and compare variation in the structures shapes (Rohlf, 1990).

Such analyzes propose a less speculative method on the evolution and diversification of the group and was already used to solve morphological problems in spiders, such: sympatric species identification (Wilson *et al.*2021), allometry and sexual dimorphism (Fernández-Montraveta & Marugan-Lobon, 2017; Kallal *et al.*2019) and intraspecific variation (Costa-Schmidt, & Araújo, 2010).

METHODS

Material

Material deposited in the following collections examined. Abbreviation, institution, city, country and curator are as follows: CAD = Coleção Aracnológica Diamantina, Rio Claro, São Paulo, Brazil (J.P.L Guadanucci); IBSP = Instituto Butantan, São Paulo, São Paulo, Brazil (A.D. Brescovit); LMB = Leando Malta Borges Private collection, Santa Maria, Rio Grande do Sul, Brazil (L.M. Borges); MCN = Museu de Ciências Naturais, Fundação Zoobotânica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil (R. Ott); MCTP = Museu de Ciências e Tecnologia, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil (R. A. Teixeira); MZSP = Museu de Zoologia da Universidade de São Paulo, São Paulo, Brazil (R. Pinto-da-Rocha).

Species identification

We used the diagnosis proposed by Bertani 2001 to identify *P. longisternalis* (Figure 2) and *P. roseus* (Figure 3). We identified as *P. longisternalis* males and females with sternum much longer than wide (Figure 4) and males with long branches of the tibial apophysis. For *P. roseus* we identified males and females with shorter sternum, males with short branches of the tibial apophysis and females with long setae on the ventral side of the femur and coxae. Furthermore, we noticed that some individuals of *P. roseus* have a sternum with a longer aspect than other conspecifics. Based on the ratio length/width, we considered as *P. roseus* morpho1 all individuals below or equal to that of the median value (Figure 5), and as *P. roseus* morpho2 those above the median value (Figure 6).

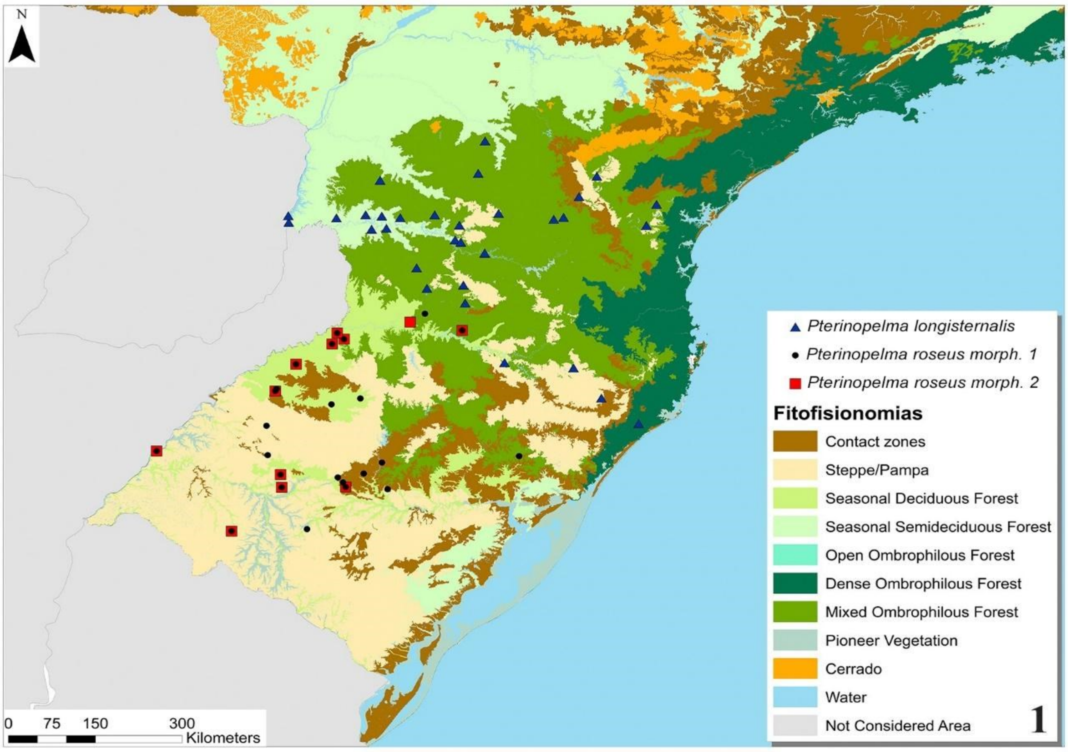
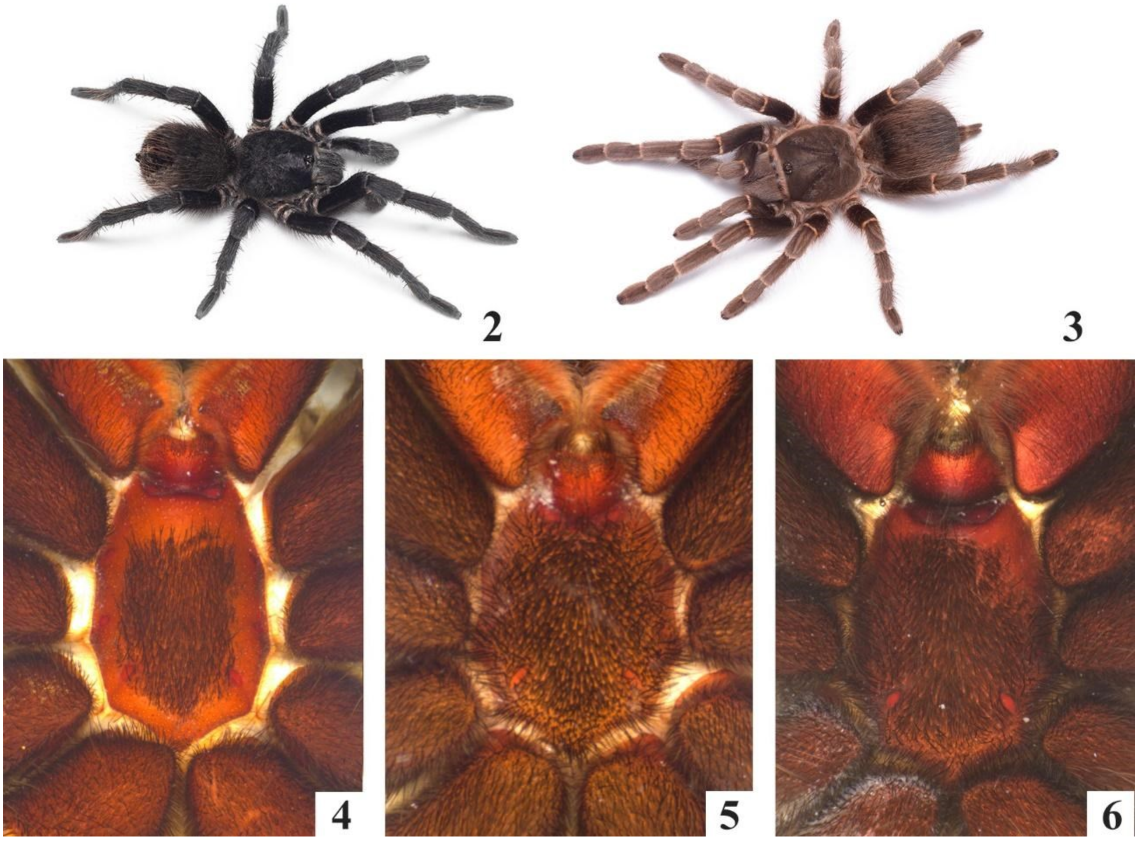


Figure 1. Distribution map of *P. longisternalis* and *P. roseus* in southern Brazil.



Figures 2-6. Differences between the sternum of *P. longisternalis* and *P. roseus*. **2:** *P. longisternalis* (CAD PR2022The3) mature male. **3:** *P. roseus* (LMB018) mature female **4:** *P. longisternalis* (MZSP17943) female. **5:** *P. roseus* (LMB209) female (morphotype 01). **6:** *P. roseus* (LMB 053) female (morphotype 02).

Analysis and Procedures for Morphometric Analysis

Photographs and measurements (given in millimeters) were taken using a Leica MC170 digital camera mounted on a Leica M205C stereomicroscope with a LAS Core software v4.12.0. All structures were photographed on a white background, centered on the frame and in the same orientation. For the sternum, we used 1x lens (model 10450028) at 1.25x magnification. For the male palpal bulbs, we photographed the prolateral view with a 1x lens (model 10450028) at 2.5x magnification. For the female spermathecae, we photographed the dorsal view with a 1x lens (model) at 3.2x magnification. All the palpal bulbs and spermathecae were immersed in 70% alcohol gel because the density of this fluid allows the homologous positioning of the structures. Immature individuals and those with damaged structures were not included in the analyses.

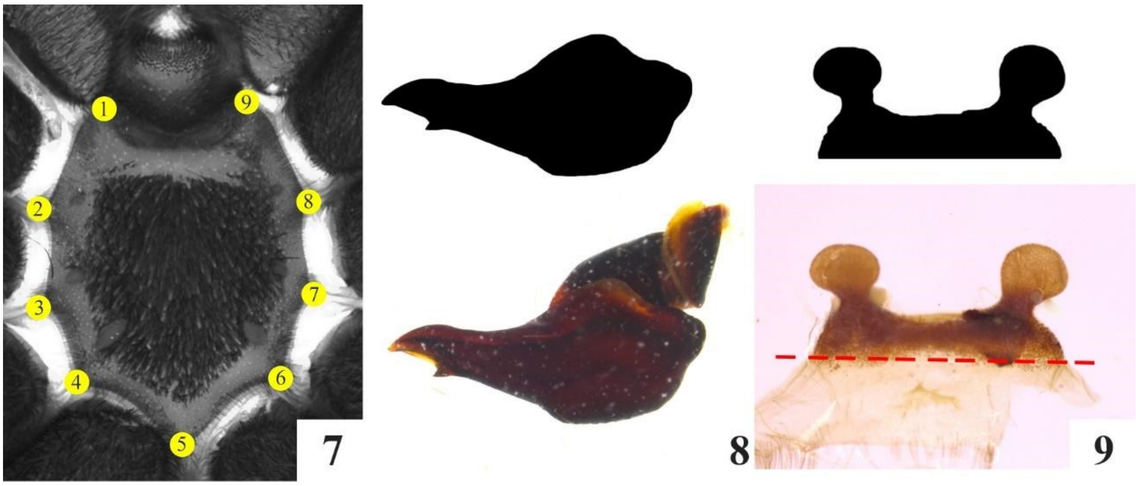
Image Processing for Analysis

The images of the sternum were grouped in a single folder and converted to TPS format using the tps-Util 1.28 software (ROHLF, 2004). For the insertion of the anatomical landmarks, we used the software TPSDIG32 *P.*1.31 (ROHLF, 2001). Nine landmarks were used for the analysis of sternum (Figure 7). The descriptions and types of landmarks are in Table 2.

Table 2. Type and description of the sternum landmarks used in the geometric morphometric analysis of *P. longisternalis* and *P. roseus.*

Landmarks	Type	Description
1	I	Insertion area of the left endite/left basal edge of the labium.
2	I	Insertion area of left coxae I.
3	I	Insertion area of left coxae II.
4	I	Insertion area of left coxae III.
5	I	Insertion area of left and right coxae I*P*.
6	I	Insertion area of right coxae III.
7	I	Insertion area of right coxae II.
8	I	Insertion area of right coxae I.
9	I	Insertion area of the right endite/right basal edge of the labium.

To highlight the contours of the male palpal bulbs and female spermathecae, we used the Adobe Photoshop CS6 software to extract the silhouettes using the magnetic lasso and eraser tool. For the bulbs, we outlined the tegulum and embolus region, including keels. For the spermathecae, we used the receptacles and the sclerotized base, cutting at the point where the sclerotization ends. Then we filled the interior of the contour with black, changed the color type to gray scale and flattened the images to a white background (Figures 7-9).



Figures 7-9. Morphological characters used for morphogeometric analyzes between *P. longisternalis* and *P. roseus*. **7:** *P. roseus* (MCN4524) female, insertion points of landmarks. **8:** *P. longisternalis* (CAD1240) male palpal bulb, after and before silhouette extraction. **9:** *P. longisternalis* (CAD1214) female spermathecae, after and before silhouette extraction. Red dashed line indicates the cut made at the end of sclerotization area.

Geometric Morphometric and Elliptic Fourier Analysis (EFA)

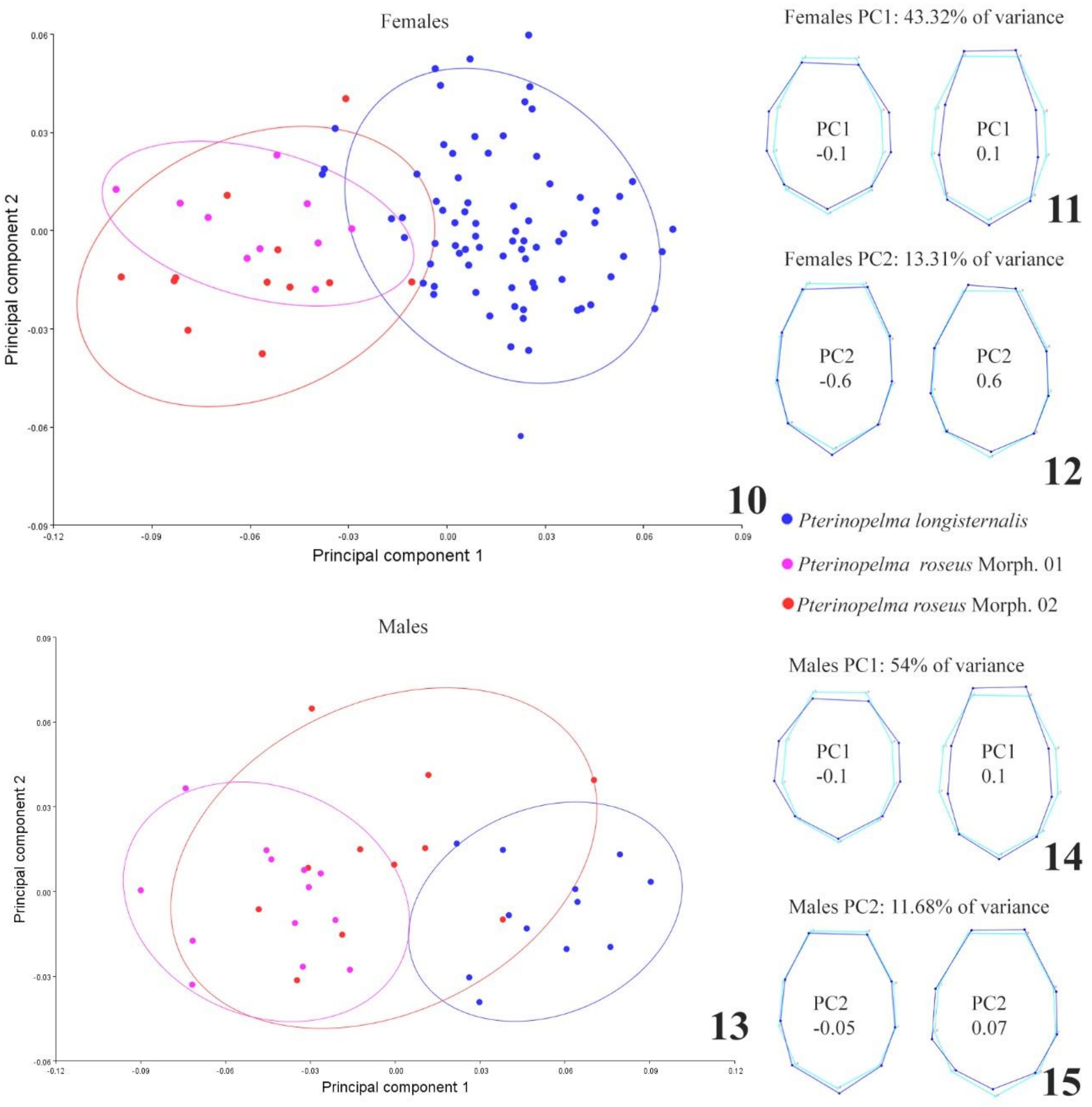
For the geometric morphometric analysis of the sternum, we use MorphoJ *P.*1.05 software (Klingenberg, 2011). We analyzed males and females separately in order to remove the influence of sexual dimorphism on the results. First, we conducted a Generalized Procrustes Analysis (GPA) to eliminate the effects of size, position and orientation. After, a covariance matrix was extracted, and principal component analyzes (PCA) were performed to identify the main variations that explain the shape configurations and the distribution of data in the morphospaces of the PCs. To correct possible allometry effects in the data distribution, we performed a regression analysis using the procrustes coordinate as a variable dependent and centroid size as a predictor variable. In addition, we perform tests against the null hypothesis of independence (10,000 iterations). For the recovery of the groups, we conducted an Analysis of Canonical Variables (CV) and discriminant functions (DF) with cross validation, considering the significance of the result through the p-value of Malahanobis distances and Procrustes distance. To visualize the differences in the ratios between the length and width of the sternum, we made a boxplot with the values and conducted an ANOVA in R environment software (R Core Team, 2016).

EFA was performed using the R environment software (R Core Team, 2016), using the packages: “Geomorph”, “Momocs” and “Morpho” (Bonhomme *et al.*, 2014; Schlager, 2017; Adams *et al.*, 2021). We first eliminate the influences of rotation, size and orientation of the images and after aligning, we searched for the necessary number of harmonics to recover the maximum contour of each structure (maximum Fourier power). After that, a PCA was performed to find the components that better explained the variation of shapes. We analyzed both species separately to compare the average axes of PC1 and then overlapped the two forms, visualizing and identifying the variation between each species through a grid and heat map. Finally, we conducted a discriminant analysis to retrieve the groups according to the variation observed in the contour analysis.

RESULTS

Morphometric results

For the sternum analysis, we used 89 specimens of *P. longisternalis* (11 males and 78 females) and 48 *P. roseus* (25 males and 23 females). After Procrustes fit, we extract the allometry effect using a Regression Analysis. The results showed a significant effect of 5.44% allometry for females (p-value: 0.0011) but not significant for males with 4.17% allometry (p-value: 0.1828). The PCA based on the covariance matrix with the regression residuals showed a variance of 43.32% for PC1 and 13.31% for PC2 in females (Figure 10) and 54% for a PC1 and 11.68% for PC2 in males (Figure 13). More than 80% of the accumulated variation was concentrated in the six first components in both sexes. Still, only the PC1 and PC2 presented significance greater than 10%. The PCs shapes and distribution of data are in Figures 10-15.



Figures 7-12. Principal components of the covariance matrix of male and female *P. longisternalis* and *P. roseus* datasets. **7:** Morphospace of PC1 x PC2 for females dataset. **8:** Females PC1 variance with minimum and maximum shape variation. **9:** Females PC2 variance with minimum and maximum shape variation. **10:** Morphospace of PC1 x PC2 for males dataset. **11:** Males PC1 variance with minimum and maximum shape variation. **12:** Males PC2 variance with minimum and maximum shape variation.

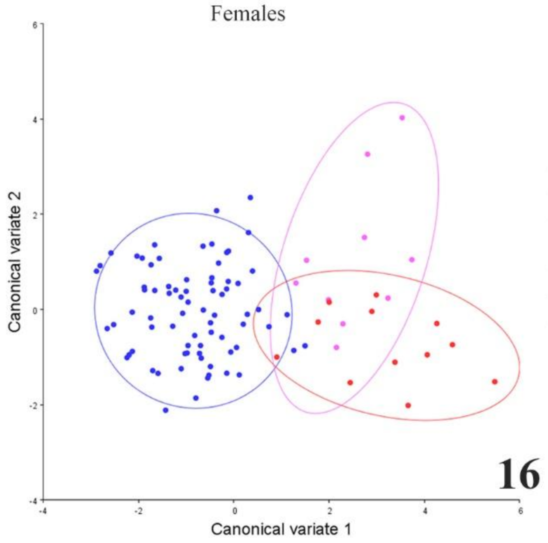
Figure 16 and 17 corresponds to CVA of female and male specimens. In females CVA, the Mahalanobis distances between *P. longisternalis – P. roseus* morpho1 is 3.5589 (p-value < 0.0001), *P. longisternalis – P. roseus* morpho2 is 4.1196 (p-value < 0.0001), and between the two morphotypes of *P. roseus* is 1.9473 (p-value: 0.0772). The distances of procrustes were significant comparing *P. longisternalis* with the two morphotypes of *P. roseus* (p-value < 0.0001 in both cases), but between *P. roseus* morphotypes the p-value is not significant (0.5632).

In Males CVA, the Mahalanobis distances between *P. longisternalis – P. roseus* morpho1 is 5.0949 (p-value < 0.0001), *P. longisternalis – P. roseus* morpho2 is 4.0292 (p-value < 0.0001) and between the two morphotypes of *P. roseus* is 2.9866 (p-value < 0.0001). The procrustes distances were significant in all comparisons (p-value < 0.0001 *P. longisternalis – P. roseus* both morphotypes, p-value 0.0022 between *P. roseus* morphotypes).

In the discriminant analysis (DA) tests, for both sexes, the recovery rates (Procrustes distances and T-square) were significant between *P. longisternalis* compared to both morphotypes of *P. roseus* (p-values: procrustes distance < 0.0001, T-square < 0.0001). However, among the morphotypes of *P. roseus*, recovery rates were not significant (females p-values: procrustes distance 0.4460, T-square 0.4340; males p-values: procrustes distance < 0.0001, T-square 0.1420). The DA results are in Table 2-3.

To complement the results of the geomorphological analyses, we tested the significance of the differences sternum length-to-width ratios (Figure 18) for both species using ANOVA (F = 256.4, p < 0.05).

Canonical Variate Analysis



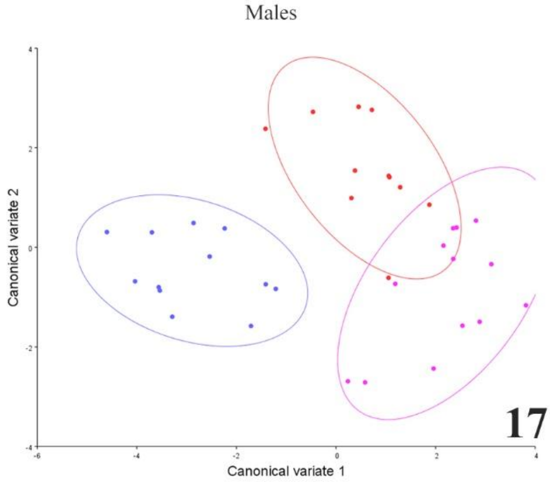
16

Malahanobis Distance:
P. longisternalis - *P. roseus* Morph. 1 - 3.5589 (p-value <0.0001)
P. longisternalis - *P. roseus* Morph. 2 - 4.1196 (p-value <0.0001)
P. roseus Morph. 1 - *P. roseus* Morph. - 1.9473 (p-value 0.0772)
Procrustes Distance:
P. longisternalis - *P. roseus* Morph. 1 - 0.0750 (p-value <0.0001)
P. longisternalis - *P. roseus* Morph. 2 - 0.0771 (p-value <0.0001)
P. roseus Morph. 1 - *P. roseus* Morph.2 - 0.0187 (p-value 0.5632)

● *Pterinopelma longisternalis*

● *Pterinopelma roseus* Morph. 01

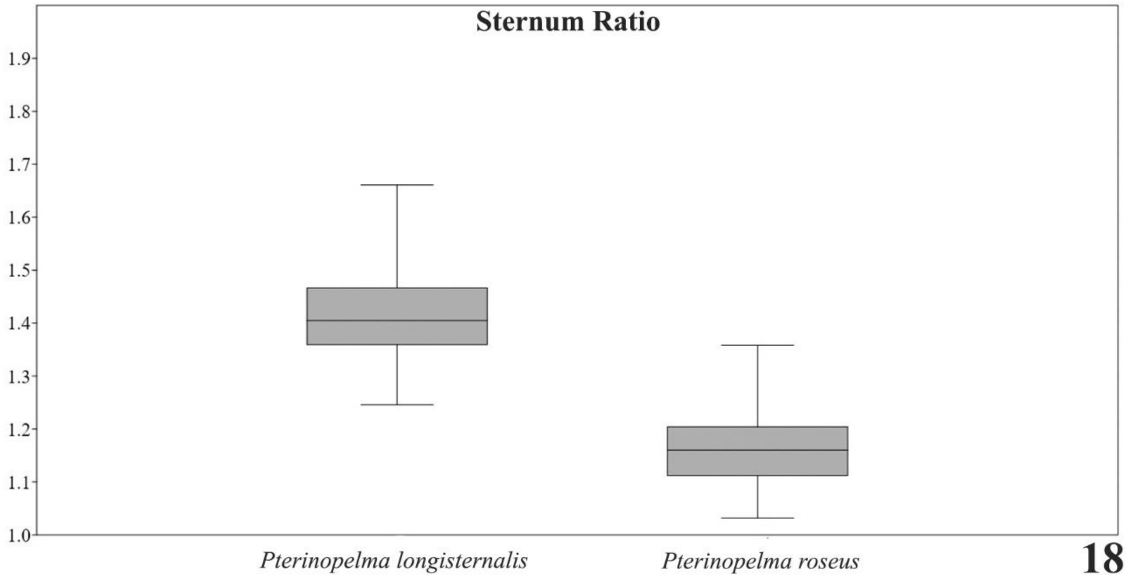
● *Pterinopelma roseus* Morph. 02



17

Malahanobis Distance:
P. longisternalis - *P. roseus* Morph. 1 - 5.0949 (p-value <0.0001)
P. longisternalis - *P. roseus* Morph. 2 - 4.0292 (p-value <0.0001)
P. roseus Morph. 1 - *P. roseus* Morph. - 2.9866 (p-value <0.0001)
Procrustes Distance:
P. longisternalis - *P. roseus* Morph. 1 - 0.0991(p-value <0.0001)
P. longisternalis - *P. roseus* Morph. 2 - 0.0621 (p-value <0.0001)
P. roseus Morph. 1 - *P. roseus* Morph. 2 - 0.0465 (p-value 0.0022)

Figures 16-17 Canonical Variable Analysis of male and female *P. longisternalis* and *P. roseus* datasets. **16:** Results of CVs for females. **17:** Results of CVs for males.



Figures 18 Boxplot of the ratio values between length and width of the sternum of *P. longisternalis* and *P. roseus*.

Table 2. Results of Discriminant Analysis (DA) and Cross Validation (CV) for females of *P. longisternalis* and *P. roseus* morphotypes 1 and 2. The lines indicate the species of analyzed specimens and the columns indicate the species compared. The values represent the total of specimens recovered and the percentage of recovery. P-values for *P. longisternalis* x *P. roseus* both morphotypes: procrustes distance < 0.0001, t-square < 0.0001. P-values between *P. roseus* morphotypes: procrustes distance 0.4460, t-square 0.4340.

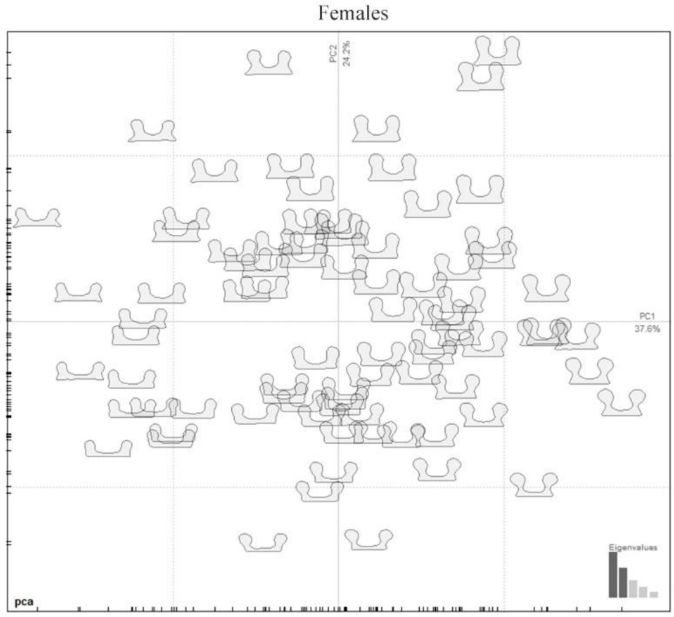
FEMALES						
	P. longisternalis		***P. roseus* morpho1**		***P. roseus* morpho2**	
	DA	**VC**	**DA**	**VC**	**DA**	**VC**
P. longisternalis	-	-	73/75 - 97%	71/75 – 94%	73/75 - 97%	72/75 – 96%
***P. roseus* morpho1**	10/10 – 100%	9/10 – 90%	-	-	9/10 – 90%	4/10 – 40%
***P. roseus* morpho2**	10/12 – 91%	10/12 – 83%	12/12 – 100%	5/12 – 41%	-	-

Table 3. Results of Discriminant Analysis (DA) and Cross Validation (VC) for males of *P. longisternalis* and *P. roseus* morphotypes 1 and 2. The lines indicate the species of analyzed specimens and the columns indicate the species compared. The values represent the total of specimens recovered and the percentage of recovery. P-values for *P. longisternalis* x *P. roseus* both morphotypes: procrustes distance < 0.0001, t-square < 0.0001. P-values between *P. roseus* morphotypes: procrustes distance < 0.0001, t-square 0.1420.

MALES						
	P. longisternalis		***P. roseus* morpho1**		***P. roseus* morpho2**	
	DA	**VC**	**DA**	**VC**	**DA**	**VC**
P. longisternalis	-	-	12/12 - 100%	11/12 – 91%	12/12 - 100%	9/10 – 75%
***P. roseus* morpho1**	12/13 – 100%	11/13 – 84%	-	-	12/13 – 92%	8/13 – 61%
***P. roseus* morpho2**	11/11 – 100%	9/11 – 69%	11/11 – 100%	6/11– 54%	-	-

Elliptic Fourier Analysis Results

We recovered 99.9% of the contour using 21 harmonics for the bulbs and 14 harmonics for the spermathecae. The PCA analysis for palpal bulbs shows that the first two PCs were sufficient to explain more than 70% of the variation (PC1 56.83%, PC2 13.90%). For spermathecae, the first three PCs explain 70% of the variation (PC1 37.56%, PC2 24.25%, PC3 13.88%). The results of PCs for males and females and the morphospaces are presented in Figures 19-22.



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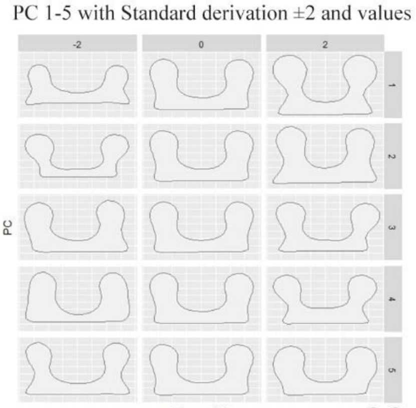
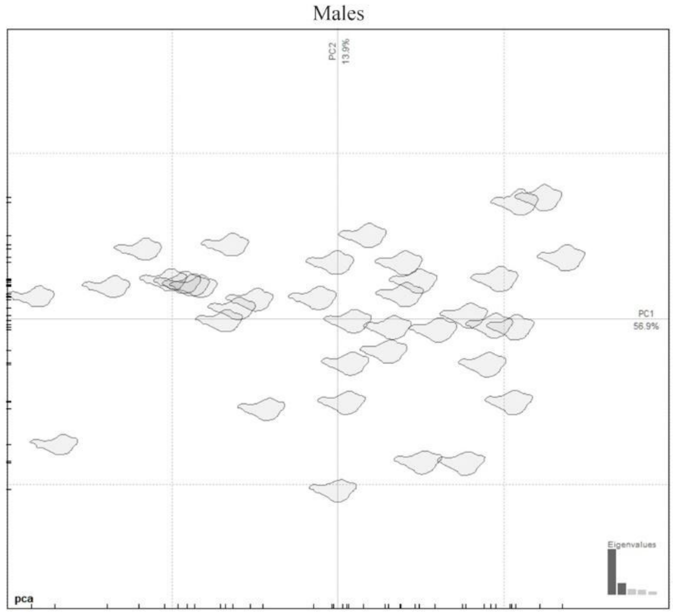


20

```
      proportion cummulative
PC1   37.564173     37.56417
PC2   24.255308     61.81948
PC3   13.889766     75.70925
PC4    8.447284     84.15653
PC5    4.267545     88.42408
```



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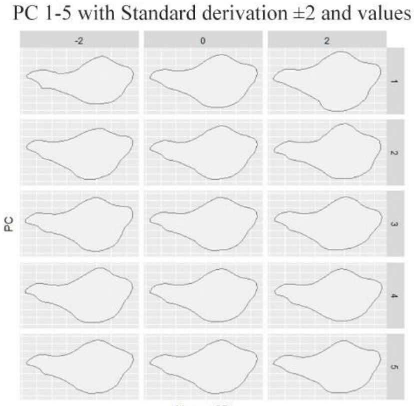
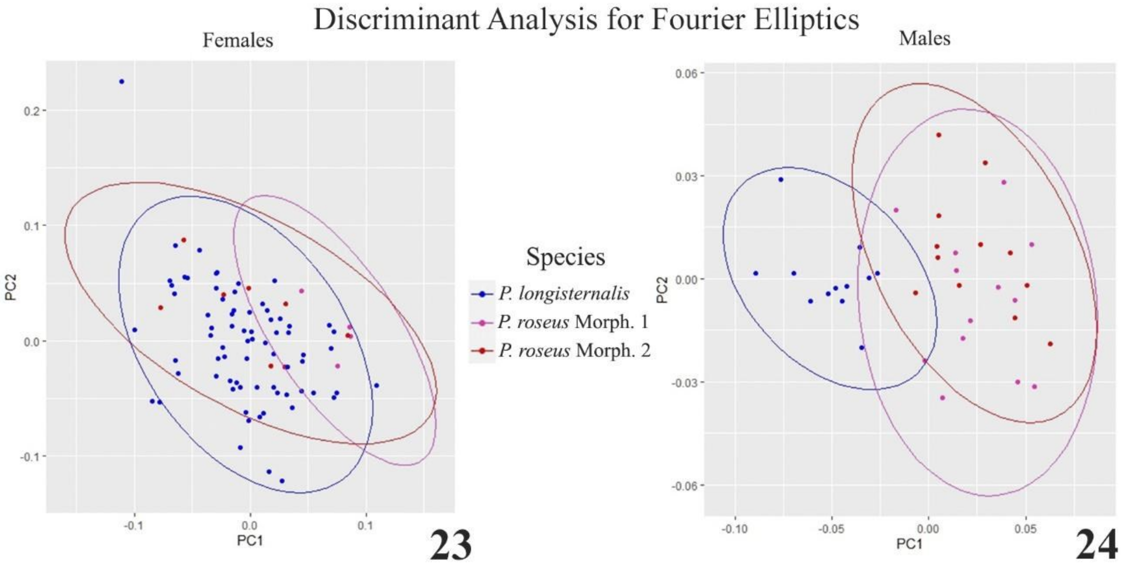


22

```
      proportion cummulative
PC1   56.836187     56.83619
PC2   13.902043     70.73823
PC3    7.308353     78.04658
PC4    5.344420     83.39100
PC5    4.077069     87.46807
```

Figures 19-22 Principal components of Elliptic Fourier Analysis of palpal bulbs and spermathecae of *P. longisternalis* and *P. roseus*. **19:** Morphospace of PC1 x PC2 for females spermathecae. **20:** Males PC1 to PC5 Shape variance with standard variation ±2 and values. **21:** Morphospace of PC1 x PC2 for males palpal bulbs. **22:** Males PC1 to PC5 Shape variance with standard variation ± 2 and values.

A linear discriminant analysis was performed with the data from the normalized Fourier coefficient matrix. The results of the retrievals and visualizations of the groups are shown in Figures 23-24.

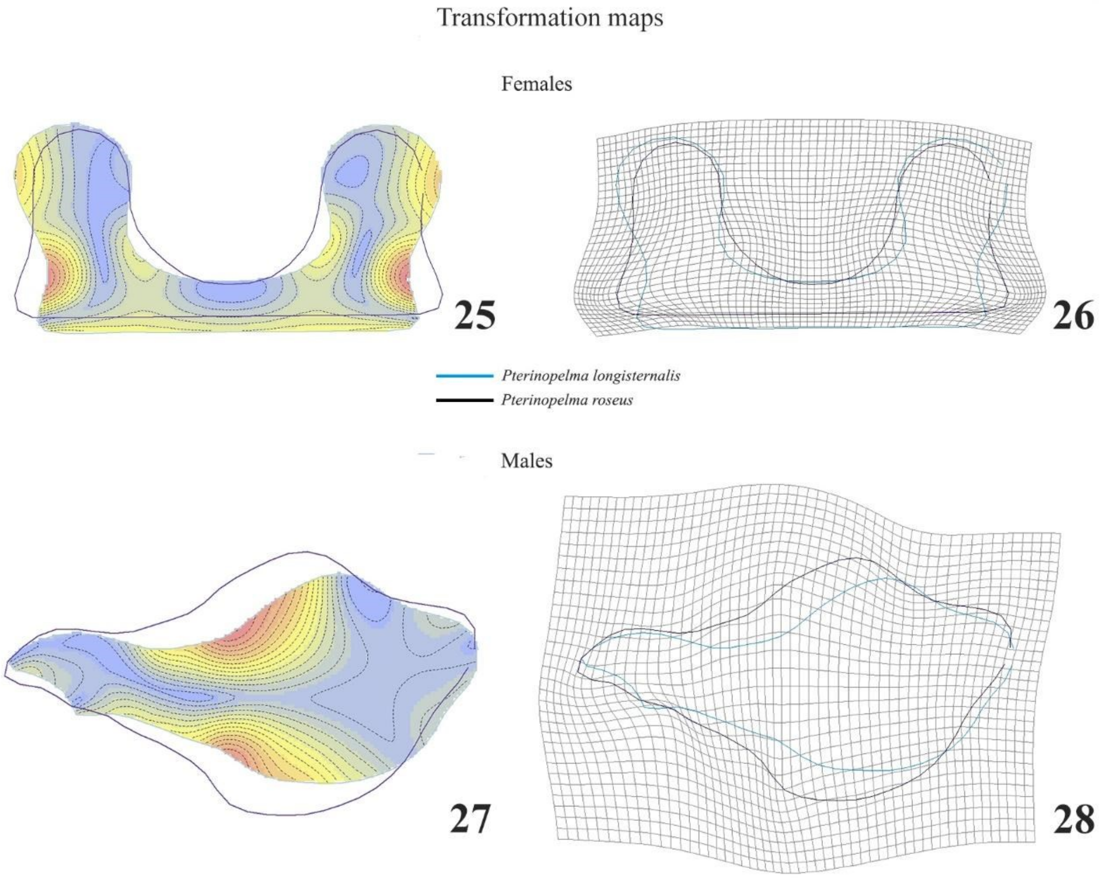


	P.lon.	*P.ros.* M1.	*P.ros.* M2.
P.lon.	69	0	1
P.ros. M1.	4	3	0
P.ros. M2.	2	0	5

	P.lon.	*P.ros.* M1.	*P.ros.* M2.
P.lon.	8	2	2
P.ros. M1.	3	7	3
P.ros, M2.	5	5	2

Figures 23-24 Discriminant analysis of palpal bulbs and spermathecae of *P. longisternalis* and *P. roseus*. **23:** Discriminant analysis results for females spermathecae. **24:** Discriminant analysis results for males palpal bulbs.

We also performed a Fourier analysis separately between *P. longisternalis* and *P. roseus* to obtain the average PC1 value of each species. Then, we compared the shape of this value between species to observe possible variations between bulbs and spermathecae. The results of these analyzes are shown in figures 25-28.



Figures 25-28 Transformations maps between palpal bulbs and spermathecae of *P. longisternalis* and *P. roseus*. **25:** Heat map of the variation between spermathecae of *P. longisternalis* and *P. roseus*. **26:** Transformation grids between spermathecae of *P. longisternalis* and *P. roseus.* **27:** Heat map of the variation between palpal bulbs of *P. longisternalis* and *P. roseus*. **28:** Transformation grids between palpal bulbs of *P. longisternalis* and *P. roseus.*

DISCUSSION

When analyzing the shape variation between the sternum of *P. longisternalis* and *P. roseus*, it is possible to observe that the main variations occur in the axes of width and length. Regardless of the CVA and discriminant functions have shown significant recovery values for males and females of *P. longisternalis* from *P. roseus*, the same was not verified between the morphotypes of *P. roseus*. In addition, it was observed in both species that this structure suffers an allometric effect in females, but not in males. We suspect this is a result of the continued growth of females after sexual maturation (Baerg & Peck, 1970; Maki, 1989), something that does not happen in males. However, due to the lower sample size of males in our data, further analyzes should be performed to confirm this hypothesis.

When we compare the distribution of the ratios whit the CV results, is possible to observe a partial overlap in the data, suggesting that this character has a continuous aspect in females. However, given the total separation in males and the partial overlap in females, we infer that the "sternum length" in *P. longisternalis* is indeed diagnostic. A way to describe this character in a specie diagnosis is by the median values of the ratios. So, we propose that *P. longisternalis* differs from *P. roseus* by the sternum ratio which is 1.42 ± 0.11 for *P. longisternalis* and 1.17 ± 0.07 for *P. roseus*.

The results of EFA of sexual characters (palpal buls and spermathecae) indicated a significant variation in male palpal bulbs between the species. *P. longisternalis* has a dorso-ventral flattening at the base of the embolus, giving this structure a thinner and longer appearance, compared to that of *P. roseus*. Furthermore, our discriminant analysis was able to recover *P. longisternalis* from *P. roseus*, but there was no distinction between bulbs of the two proposed morphotypes of *P. roseus*. For females, the EFA results showed that the receptacles ducts of the spermathecae present highly variance (Fig 25-26), This variation covers most of the morphospace (Fig. 19) and, thus, not forming cluster.

It is expected a greater variation in female structures than in males, since in Theraphosidae, females are responsible for sexual selection (Costa *e al.*, 2013; Pérez-Miles & Perafán, 2017). Therefore, male structures would accumulate fewer modifications and consequently preserve a standard form to warrant conspecific recognition by the female. Thus, the variation in the shape of the spermathecae is not strong enough to differentiate the two species.

CONCLUSION

Our analyzes were successful in recovering males and females of *P. longisternalis* from *P. roseus* based in differences on sternum of both sexes and males palpal bulb. Also, the uses of the median values of the sternum ratio between the two species shows useful to describe the result of the morphometric analysis in a species diagnosis. These values are also useful for encoding this character into a phylogeny analysis. However, the taxonomic differentiation of females is still a challenge because of the high heterogeneity of characters, especially the spermathecae.

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Capítulo 2

Genetic approach in populations of *Pterinopelma longisternalis* (Bertani, 2001) and *Pterinopelma roseus* (Mello-Leitão, 1923).

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Abstract:

Compared to other spider infraorders**,** the systematics of Mygalomorphae spiders is historically considered problematic**.** The group is marked by its conserved morphology, especially among close species. The advancement of molecular technique shows that, some molecular markers are effective in recovering species and evolutionary patterns. the genus *Vitalius* is a example of divergence between molecular and morphological relationship hypothesis. Recently phylogenomic analyses propose the non-monophyly of *Vitalius* and suggest that *Pterinopelma longisternalis* is the sister of *Pterinopelma roseus* and form a separate clade with *Pterinopelma vitiosum*. However morphometric analysis pointed that the variations in diagnostical characters between *P. longisternalis* then *and P. roseus* is continuous and this species cannot be empirically distinguished. We conducted a molecular investigation to identify intra- and inter-specific genetic variation and explore the existence of cryptic species in the group. Our analyzes show the existence of a new lineage between these two species, closely related to *P. longisternalis*. Furthermore, we identified intraspecific lineages for both species. The results of this papper reinforce the hypothesis that morphological differentiation in Mygalomorphae species does not follow genetic diversification, and integrative analyzes can be a more effective method of describing evolutionary patterns in Mygalomorphae spiders.

INTRODUCTION

The systematics of Mygalomorphae spiders is historically considered chaotic and problematic(Raven, 1985). Compared to other spider infraorders, Mygalomorphae is a group marked by its conserved morphology, especially among related species (Satler *et al.*2013) such features can generate homoplastic reconstructions, based on characters with continuous variations (Bond & Opell, 2002; Pérez-Miles & Perafán, 2020). The family Theraphosidae is the most diverse of the Mygalomorphae, represented by 1055 species(World Spider Catalog, 2023).

With the advancement of molecular biology, these techniques has constantly applied in phylogenetic and biogeographic studies using Theraphosidae spiders (Hamilton *et al.*2011, 2016; Wilson *et al.*, 2013; Hendrixson *et al.*, 2015; Ortiz & Francke, 2016; Montes de Oca *et al.*, 2016, Turner *et al.*, 2018; Hüsser, 2018; Foley *et al.*, 2019; Mendoza & Francke, 2020; Candia-Ramírez & Francke, 2021).

These studies have shown that some molecular markers are effective in recovering species and evolutionary patterns for the group, especially the mitochondrial marker Cytochrome Oxidase 1 (CO1), widely used as DNA barcoading in several animal groups (Folmer *et al.*1994; Hamilton *et al.*2011). Molecular results have also shown greater diversity compared to using morphological characters, revealing cryptic or pseudoscryptic species (Hamilton *et al.*2016; Candia-Ramírez & Francke 2021).

An example of divergences between molecular and morphological phylogenies is presented between the genus *Vitalius* Lucas Silva & Bertani 1993 and *Pterinopelma* Pocock, 1901. The first phylogenetic proposal for *Vitalius* was proposed by Bertani (2001). In this paper, the author suggests the monophyly of the group based on the morphological character and suggests *Vitalius* as sister genus of *Nhandu* Lucas 1981. At another time, Bertani *et al.* (2011) revalidate the genus *Pterinopelma* and present a relationship hypothesis based on morphological characters, which supports the monophyly of the genus and proposes it as closely related to *Lasiodora* Koch 1850.

However, in contrast to these morphological results, Galleti-Lima *et al.* (2023) proposed a new relationship hypothesis for the group, based on phylogenomic analyses. The results from this work point the non-monophyly of *Vitalius* and *Pterinopelma* and suggest some species transference from these taxa to other genera (*e.g. Pterinopelma* *longisternalis* (Bertani, 2001), *Pterinopelma roseus* (Mello-Leitão, 1923), *Proshapalopus nondescriptus* (Mello-Leitão, 1926) and *Takeapora wacketi* (Mello-Leitão, 1923), 2023 from *Vitalius; Parvicarina felipeleitei* (Bertani & Leal, 2016), and *Lasiocyano sazimai* (Bertani, Nagahama & Fukushima, 2011) from *Pterinopelma*). These molecular results also propose *Pterinopelma* and *Vitalius* as sister genera and forms a sister clade to *Nhandu.*

Focusing on *Pterinopelma* species, *P. longisternalis* and *P. roseus* shows a confusing morphological differentiation between them. The main diagnostic characters between the two species were analyzed under morphometric analyses (Moeller *et al.* In prep.) The results pointed out that the variations in morphological states are continuous and cannot be empirically distinguished. Furthermore, the distribution range of both species is close, and the species boundary is not clear.

The *Pterinopelma* species show close geographical distribution, occurring through the Southern region of Brazil along the Mixed Ombrophylous Forest, Deciduous Forest and grassland areas (Moeller *et al*, In. Prep.; Bertani *et al.*, 2011; Bertani, 2001). Also, these shows geological discontinuities witch may have influence in intra and inter specific genetic variations. The phylogenomic analysis proposed by Galleti-Lima *et al.* (2023.) did not include many representatives of each species, thus this genetic variation of these species is not tested. Given this scenario, we conducted a molecular investigation to identify intra- and inter-specific variation and a correlation between morphological variations and specie diversification.

METHODS

Material deposited in the following collections examined. Abbreviation, institution, city, country and curator are as follows: CAD = Coleção Aracnológica Diamantina, Rio Claro, São Paulo, Brazil (J.P.L Guadanucci); LMB = Leando Malta Borges Private collection, Santa Maria, Rio Grande do Sul, Brazil (L.M. Borges). We also performed fieldwork expeditions to complement distribution data and fresh material available for molecular analysis. All collected material was preserved in ethanol and deposited in the CAD collection.

Dna extraction and sequencing

The genetic material was extracted from the muscles of the femur III of the right leg of the spiders (Longhorn *et al.*, 2007). The legs and tissue were deposited in 96–100% ethanol and stored at –20 ºC. The DNA extraction was performed using the GenEluteTM extraction kit (Sigma-Aldrich), following the manufacturer's instructions. Extracted DNA was quantified with a spectrophotometer (Nanodrop 2000, Thermo Scientific, DE, USA). The DNA amplification was performed using the polymerase chain reaction (PCR) for a portion of 963 bp of the mtDNA barcoding gene cytochrome c oxidase subunit I (COI). The primers used for COI were: LCO1490 (Folmer *et al.*, 1994) and C1-N-2776 (Hedin & Maddison 2001) with the adaptations proposed by Hamilton *et al.* (2011) (Table 2).

For the PCR solutions, the Thermo Fisher Scientific Platinum™ Taq DNA Polymerase amplification kit was used, following the manufacturer's instructions, with exception of the thermocycler cycles, which was used the protocol proposed by Hamilton *et al.* (2014). The PCR result was visualised under UV light after electrophoresis in 1% agarose gel, with samples evidenced by GelRedTM. The size of the amplified fragment was estimated through comparison with a molecular weight standard sample (DNA ladder). PCR products were a quantified and diluted to 50 ng/ul in ultrapure water, purified using ExoSAP-IT (Applied Biosystems, Foster City, CA) and sent to Macrogen Inc. (Seoul, South Korea).

Table 2. CO1 primers used in the phylogenetic analyzes of *P. longisternalis* and *P. roseus*.

Primer	Reference	Sequency	Direction
LCO1490	Folmer *et al.*, (1994)	5’-GGTCAACAAATCATAAAGATATTGG-3’	foward
LCO1490aphonopelma	Hamilton *et al.* (2011)	5’-TTTCTACTAATCACAAGGATATYGG-3’	foward
LCO1490cg	Hamilton *et al.* (2011)	5’-TTTCTACTAATCACAAGGATATTGG-3’	foward
LCO1490chalcodes	Hamilton *et al.* (2011)	5’-TTTCCACAAACCACAGGGATATCGG-3’	foward
LCO1490reversum	Hamilton *et al.* (2011)	5’-TTTCTACTAATCATAGGGATATTGG-3’	foward
C1-N-2776	Hedin & Maddison (2001)	5’-GGATAATCAGAATATCGTCGAGG-3’	reverse

Phylogenetic analyzes

Sequences were manually edited with BIOEDIT 7.7.1 softwere (Hall, 1999), and aligned by MUSCLE algorithm (Edgar, 2004), using the software MEGA 11 software (Tamura *et al.*, 2021). three phylogenetic methods were performed to recover the topologies, Neighbor-Joining (NJ), Maximum Likelihood (ML) and Bayesian Inference (BI). The DNA substitution model used in our analysis is General Time Reversible +

GAMMA (GTR+G), suggested by the softwere JModelTest2 (Darriba *et al.*, 2012)following the Akaike information criterion (AIC) (Akaike 1974).

The NJ analysis, was performed in MEGA 11 software (Tamura *et al.*2021) with confidence limit for nodes calculated by the nonparametric bootstrap method (Felsenstein, 1985)with 1000 replicates. The ML was performed in the softwere RAxM-HPC *P.*8.0 (Stamatakis, 2014). Support values were obtained via ML+thorough bootstrap analysis with 100 runs of 1000 standard bootstrap replicates.

Bayesian analysis was performed with BEAST *P.*1.8.0 (Drummond *et al.*, 2012). Tree searches are conduced by 80.000.000 generations sampled every 10000. Convergence was assessed using TRACER 1.7.1. To build maximum clade credibility tree, the pakage TreeAnnotator 1.10 (BEAST package) was used, discarding 10% of generations by burn-in, as indicated by TRACER. To exploring relationships among haplotypesof *P. longisternalis* and *P. roseus*, we constructed a haplotype network though MJ algorithm using NETWORK 10 software (fluxus-engineering.com) To estimate the divergence times, the Clock model used was the Relaxed Clock Log Model using Yule process and Coalescent (constant size) tree priors and 4% pairwise divergence per million years (Hamilton *et al.*2011). As an outgroup, it used sequences from specimens of the genus *Calisoga sp.* Simon, 1889 - Nemesiidae*, Synothele* sp. Simon, 1908 – Barychelidae *e Nhandu tripepii* (Dresco, 1984) - Theraphosidae, collected from GenBank (National Center for Biotechnology Information).

RESULTS

Because some specimens were not preserved in good condition or have more than 10 years in 70% ethanol without freezeng, some of the specimens used in the morphometric analysis by Moeller *et al.* (in prep.) could not be used in the molecular analysis. Twenty-two specimens were successfully amplified, of which fourteen were identified as *P. longisternalis* and eight as *P. roseus* (Table 1), following the diagnosis proposed by Bertani (2001) adapted by Moeller *et al.* (in prep.). The alignment data comprising 929 base pair (bp) each sequence.

Table 1. List of material used in molecular analyzes.

Specie	**Voucher**	**ID**	**Locality/City**	**State**	**Country**	**Latitude**	**Longitude**
P. longisternalis	CAD2020The2	Pitl03/V03	Fernandes Pinheiro	PR	Brazil	25.475	-50.645
P. longisternalis	CAD PR2022The1	Pitl05/V05	IF Palmas	PR	Brazil	-26.511	-51.987
P. longisternalis	CAD LAB33	Pitl07/V07	Guarapuava	PR	Brazil	-25.604	-51.315
P. longisternalis	CAD PR2020The4	Pitl09/V09	Fernandes Pinheiro	PR	Brazil	-25.475	-50.645
P. longisternalis	CAD FNCHThe2	Pitl13/V13	FLONA Chapecó	PR	Brazil	-27.095	-52.776
P. longisternalis	CAD PR2020The_20	Pitl16/V16	Pitanga	PR	Brazil	-24.754	-51.766
P. longisternalis	CAD PR2022The3	Pitl17/V16	Passos Maia	SC	Brazil	-26.793	-51.960
P. longisternalis	CAD PR2022The2	Pitl18/V18	IF Palmas	PR	Brazil	-26.511	-51.987
P. longisternalis	CAD LAB68	Pitl19/V19	Guarapuava	PR	Brazil	-25.604	-51.315
P. longisternalis	CAD LAB20	Pitl20/V20	Pitanga	PR	Brazil	-24.754	-51.766
P. longisternalis	CAD SC19The1	Pitl21/V21	Urubici	SC	Brazil	-28.170	-49.527
P. longisternalis	CAD PR2020The06	Pitl23/V23	Fernandes Pinheiro	PR	Brazil	-25.475	-50.645
P. roseus	LMB433	Pitr27/V27	São Martinho da Serra	RS	Brazil	-29.536	-53.861
P. roseus	LMB330	Pitr29/V29	Santa Maria	RS	Brazil	-29.721	-53.718
P. roseus	LMB075	Pitr30/V30	Rolador	RS	Brazil	-28.188	-54.776
P. roseus	LMB043	Pitr34/V34	Jaguari	RS	Brazil	-29.481	-54.698
P. roseus	LMB399	Pitr42/V42	Santa Maria	RS	Brazil	-29.721	-53.718
P. roseus	LMB403	Pitr48/V48	Santa Maria	RS	Brazil	-29.721	-53.718
P. roseus	LMB067	Pitr51/V51	Santa Maria	RS	Brazil	-29.721	-53.718
P. roseus	LMB045	Pitr53/V53	Jaguari	RS	Brazil	-29.481	-54.698
P. longisternalis	CAD FNCHThe1	Pitl60/V60	FLONA Chapecó	PR	Brazil	-27.095	-52.776
P. longisternalis	CAD LAB258	Pitl61/V61	Rebouças	PR	Brazil	-25.621	-50.694

Tree topologies based on NJ, ML and BI analyses converge in the terminal and intermediate branches results. In general, the three analyzes pointed to the existence of a third lineage represented by specimens from Chapecó city, Santa Catarina State, Brazil (Pitl13 and Pitl60) which we will call as *Pterinopelma* sp.

Neighbor Joining and Haplotype Network results

The topology results support the differentiation between *P. longisternalis* and *V roseus* (Figure 1). A third lineage is presented, composed by vouchers Pitl13 and Pitl60, closely related to *P. roseus*. However, the support shown in this branch is the lowest in the entire analysis, 78.9 bootstrap value. The analysis showed the existence of 3 intraspecific lineages in *P. longisternalis*, all of them with support >99 bootstrap value.

The first one more basal, composed by vouchers Pitl05, Pitl17 and Pitl18, from Southeast of Paraná State and Northwest of Santa Catarina State. The second linage is composed by voucher Pitl07, Pitl19 and Pitl20 from Guarapuava city, located in the Midwest of Paraná State, close to the type locality of *P. longisternalis*. The third linage is composed by the vouchers Pitl03, Pitl09, Pitl16, Pitl21, Pitl23, Pitl61. Also, polytomies were observed in both species, probably due to the low support in the closest related branches.

Through the interpretation of the Haplotypes network (Figure 2), it is noticed that the lineage of *Pterinopelma* sp. it is almost equidistant in number of mutations from *P. longisternalis* and *P. roseus*. From a geographical perspective, *Pterinopelma* sp. has been observed in a transitional region between the distribution ranges of *P. longisternalis* and *P. roseus*. The same three linages of *P. longisternalis* recovery in the NJ topology is present in the Network results. For *P. roseus* relationships, no lineage was clearly distinguished in NJ topology, however the specimen represent by the voucher Pitr30 appears distant from the other representatives of *P. roseus*, distanced by 39 accumulated mutations.

It was also noticed that in the group composed by the specimens from the Midwest region of Paraná State, has the specimen from Urubici city (Pitl21) (Figures 1-2). The distribution point of this one is more than 500 km away from the others, in the east of the state of Santa Catarina. It was previously expected that the possible existing lineages would relate according to geographic proximity, and that the Voucher Pitl21 would stand out from the others. However, this is not the displayed result, however, this proximity is showed as a polytomy in the tree. Perhaps there are biogeographical patterns focusing on the distribution of lineages of the species, however, given the limited dataset of the analysis, it was not performed any biogeographical analysis.

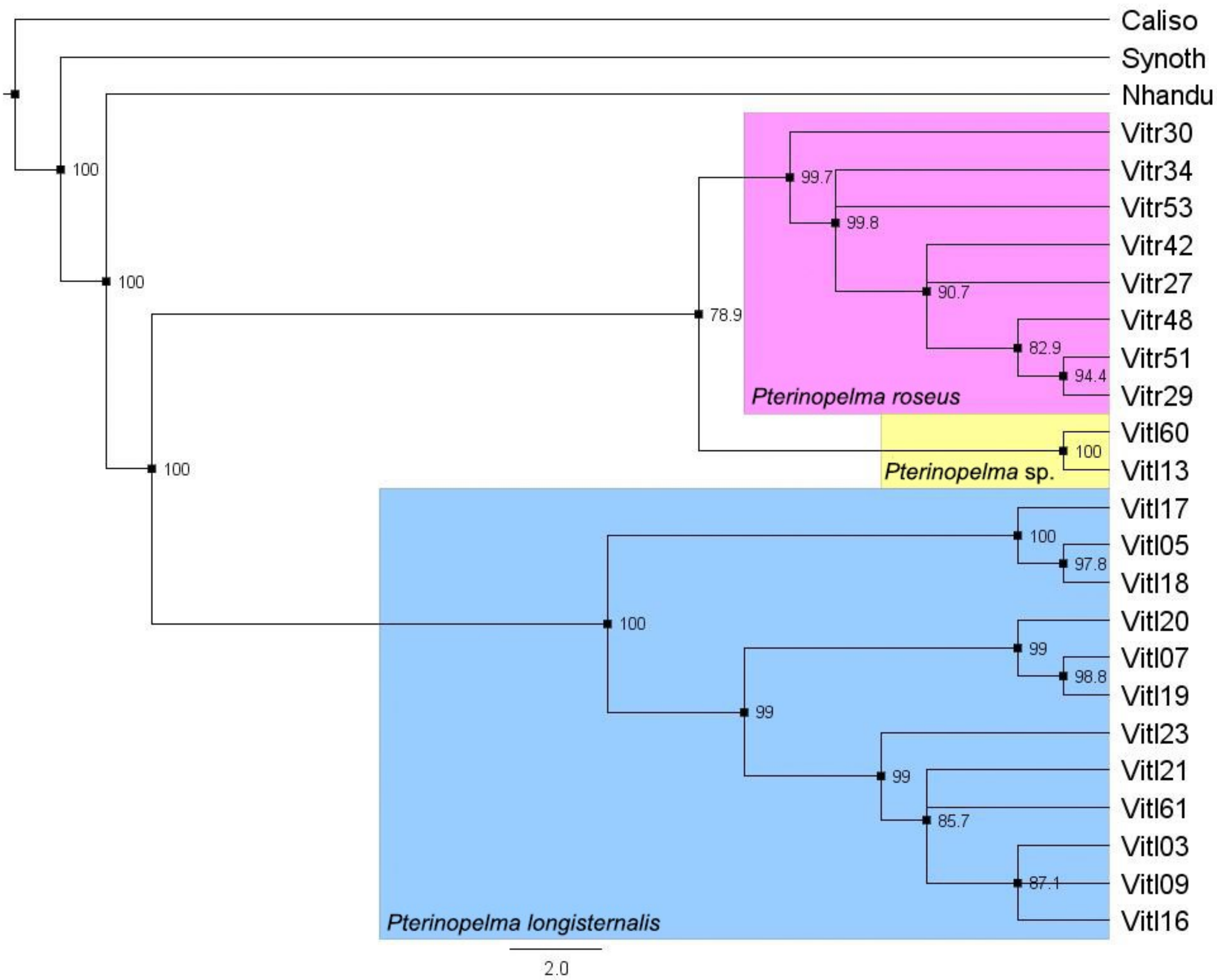

Figure 01: Neighbor Joining topology recovered from CO1 gene tree for the 22 specimens of *P. longisternalis* and *P. roseus*. Species names previously identified by morphological differentiation. Nodes squares indicates bootstrap values.

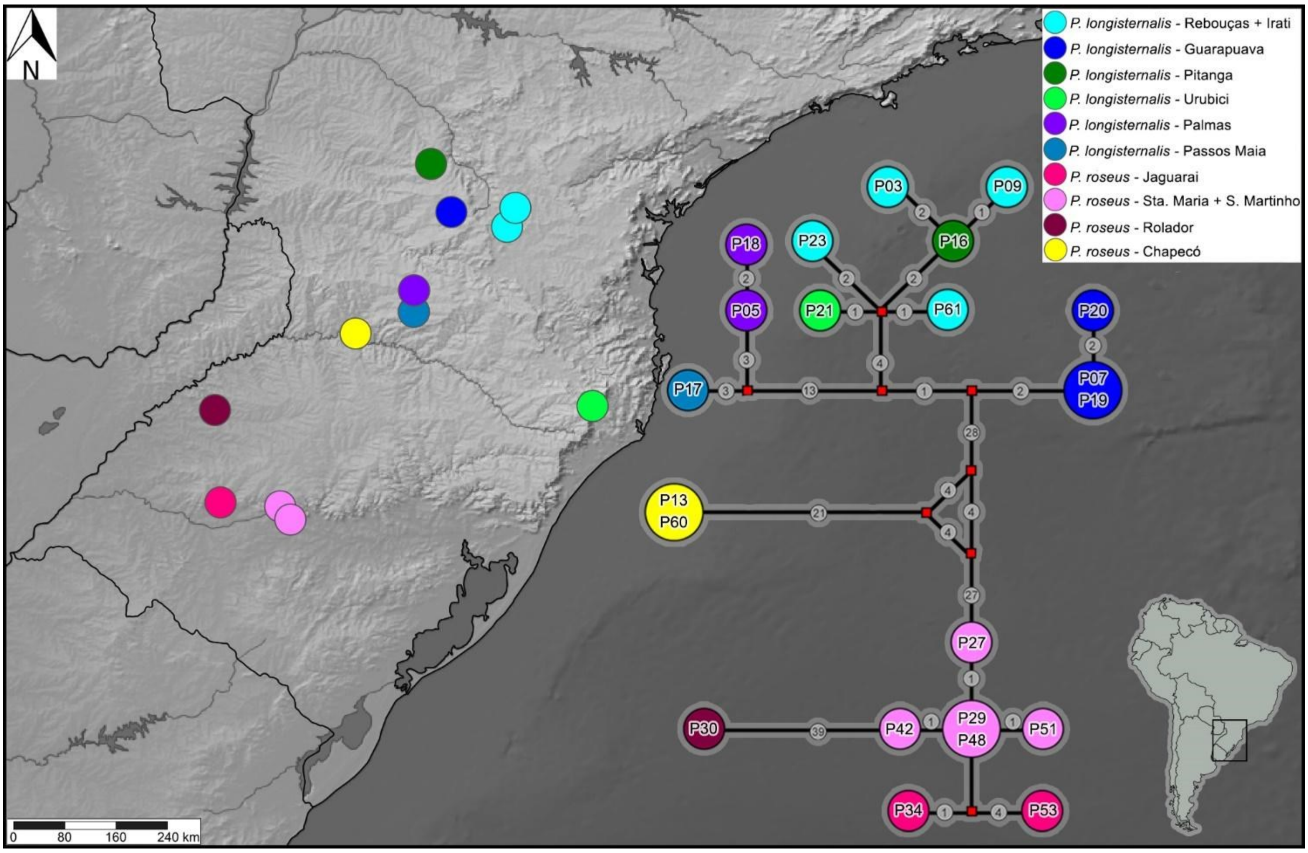

Figure 2: Network of haplotypes of the COI gene of *P. longisternalis* and *P. roseus*.

Maximum Likelihood results

Maximum Likelihood results are similar with the topology presented by the NJ analysis. This topology also discriminates *P. longisternalis* from *P. roseus* and indicates the existence of *Pterinopelma* sp. lineage, however, this is presented as a sister to the *P. longisternalis* lineage, differing from the NJ result, however, with lower support (Figure 3). The same three lineages of *P. longisternalis* observed in NJ are also observed in the topology of the ML analysis, with better supports, without presenting polytomies. For *P. roseus*, this analysis retrieved relationships more accurately, with better supported relationships.

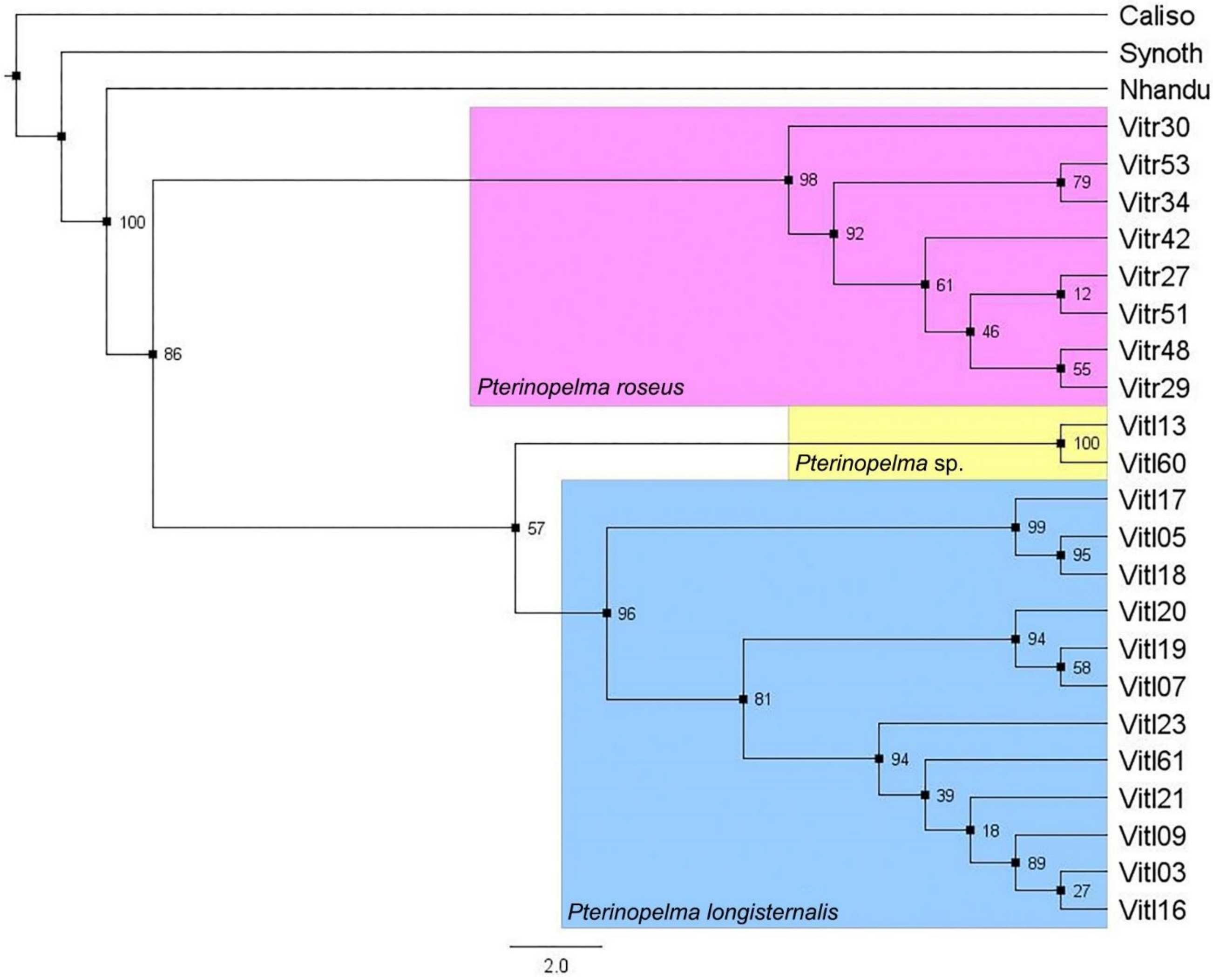

Figure 03. Maximum Likelihood topology recovered from CO1 gene tree for the 22 specimens of *P. longisternalis* and *P. roseus*. Species names previously identified by morphological differentiation. Nodes squares indicates bootstrap values.

Bayesian Inference results

The BI topology converges with the results shown in NJ and ML analysis. *Pterinopelma* sp. is identified in this analysis close as related to *P. longisternalis*, but with not significant bootstrap Value 0.75. (Figure 4, yellow box). The population lineages of *P. longisternalis* that were previously projected are supported by the BI topology with significant support (>95%). Similarly with *P. roseus* with the separation of the Pitr30 voucher from the other in the topology.

The age dating of this topology suggests recent speciation between the lineages, ca. 0.9MY between *P. longisternalis* and *P. roseus* and ca. 0.79MY between the *Pterinopelma* sp. with *P. longisternalis*. For the intraspecific lineages of *P. longisternalis*, the differentiation between the lineage from Southeast of Paraná State and Northwest of Santa Catarina State (Pitl05, Pitl17 and Pitl18) differed from the others at ca. of 0.28MY, while the other lineages diverged more recently, at ca. 0.17MY. In populations of *P. roseus*, the time of general divergence is even more recent, ca. 0.11MY except for specimen Pitr30, with the divergence time from the others is ca. 0.51MY.

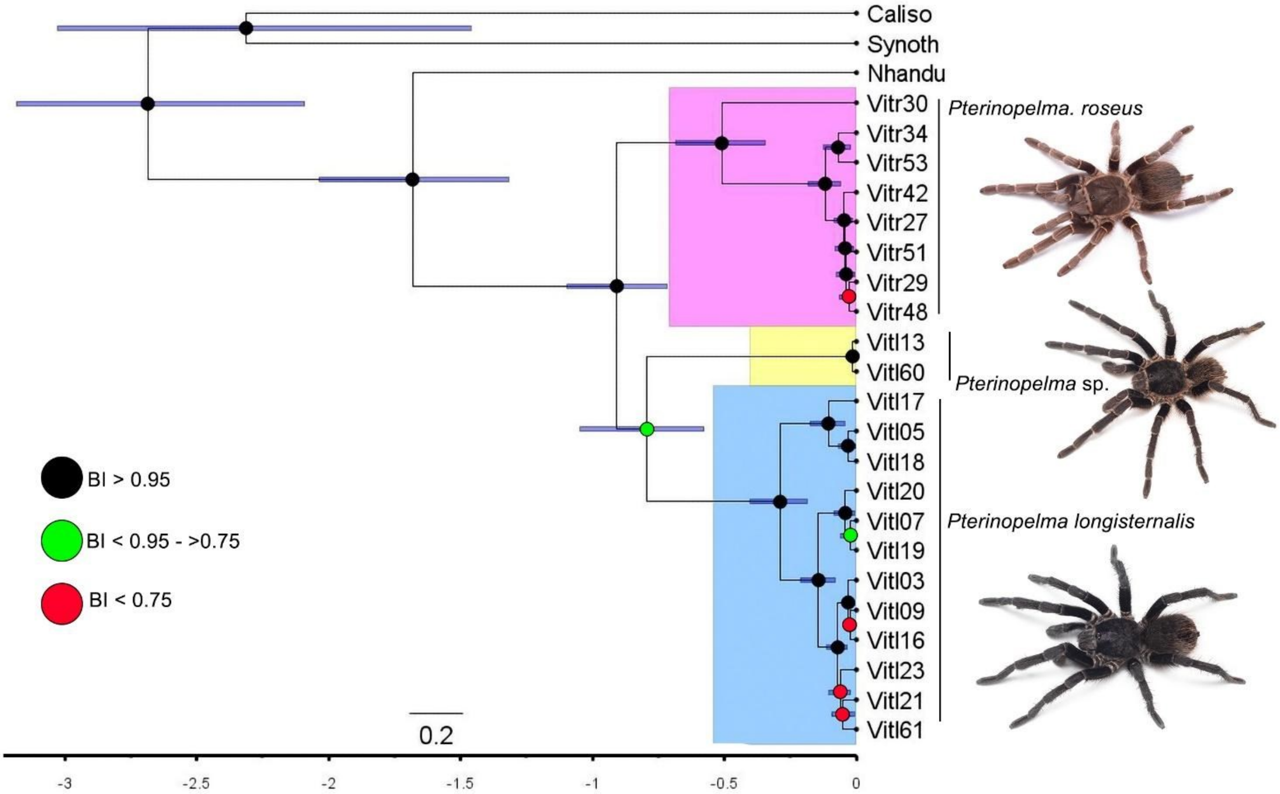

Image 04. Bayesian Inference topology recovered from CO1 gene tree for the 22 specimens of *P. longisternalis* and *P. roseus.* Species names previously identified by morphological differentiation. Colors indicate posterior values. dating calculated in millions of years. Node bars represent the 95% height posterior density.

DISCUSSION

The analysis results converge to the existence of a third lineage, closely related to *P. longisternalis* and *P. roseus*. However, the phylogenetic placement of this lineage is not conclusive. The haplpotype network results show that this lineage is equidistant in number of mutations from *P. roseus* and *P. longisternalis*, and perhaps because of this, its position is not defined. From the mophologic point of view, the new lineage can be identified as *P. longisternalis,* sharing the same diagnostical characters (Figure 5). This indicates the possibility of being a cryptic lineage. However, juveniles have an appearance similar with those of *P. roseus*.

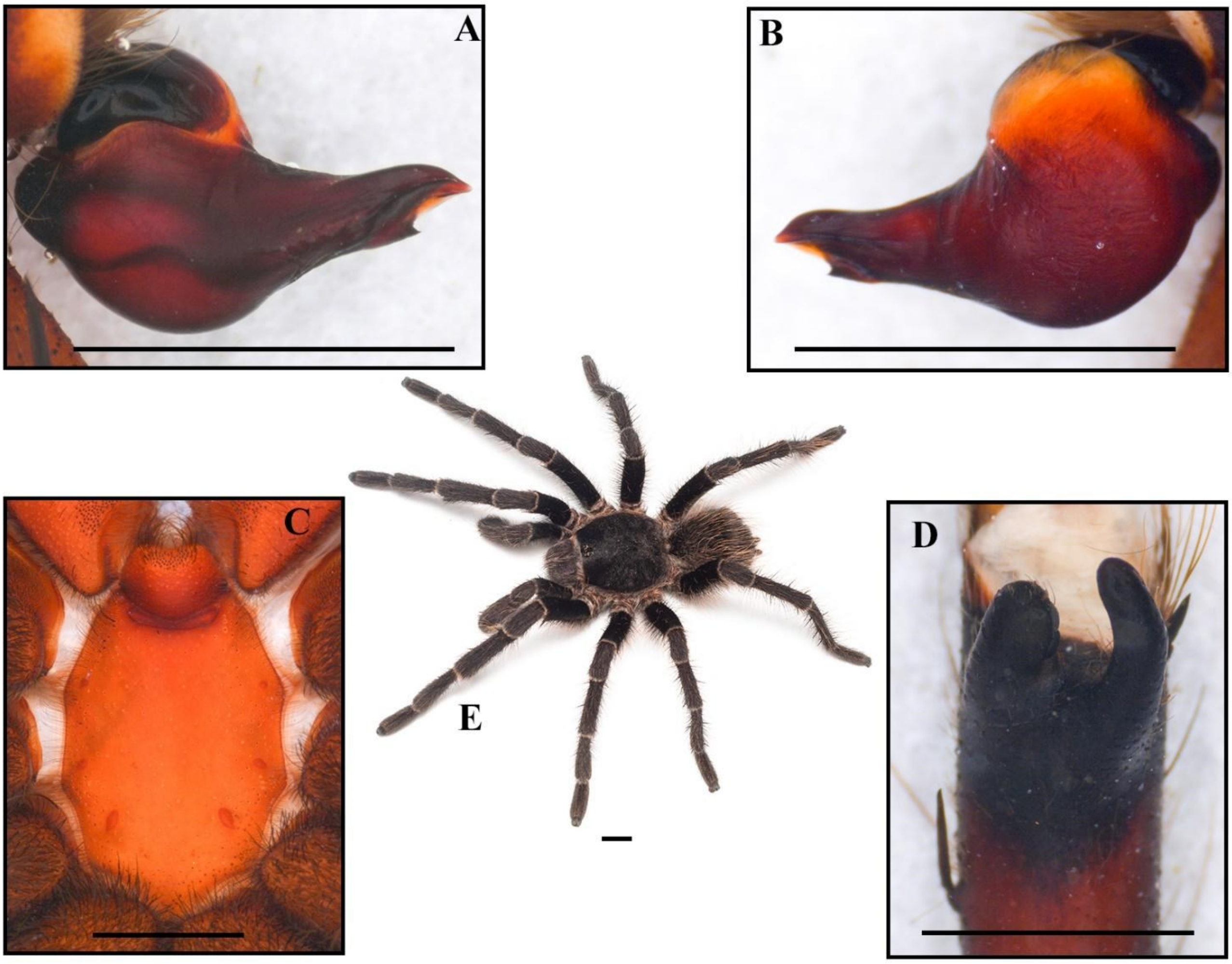

Figure 5 *Pterinopelma* sp**.** male main morphological characters (CAD FNCHThe1). **A.** Palpal bulb prolateral view; **B.** Palpal bulb retrolateral view; **C.** Sternum; **D.** Tibial apophysis; **E.** Specie general view. Scales bars = 1mm.

The point of occurrence of *Pterinopelma* sp. is in a transition region between the distribution of *P. roseus* and *P. longisternalis* (Figure 2). Given the morphological similarity and high genetic distance from *P. longisternalis*, the emergence of this clade can be originated by cryptic speciation.

A biogeographic hypothesis can be raised to explain this clade. In the past, some allopatric or peripatric events may have occurred in ancestral populations. In this case, due to the recent diversification between them, the morphological characters states have not changed. Cryptic speciation has already been reported for Mygalomorphae and Theraphosidae spider associated with allopatric or peripatric events (Hamilton *et al.* 2011, 2016; Satler *et al.* 2013; Leavitt *et al.* 2015; Ortiz & Francke 2016; Candia-Ramírez & Francke 2021; Montes de Oca *et al.* 2022). It is important notice that the distribution area of these lineages is characterized by shapeless topography, with regions of high and medium altitudes interspersed by mountains and escarpments, characteristics that may have biogeographical influence (Christofoletti, 1980, Vitte & Guerra, 2004).

However, a deep analysis capable of testing biogeographical effects or hybridization process was not possible to conduct with our data, due to limitations in the number of specimens and sampled populations. The events that may give rise to differentiation between *P. roseus* and *P. longisternalis* and the formation of their lineages can and should be tested. However, for that, an extensive data set must be examined, containing more locations sampled and these with more specimens represented. Furthermore, it was not possible to include representatives of species of *Vitalius stricto sensu* and *P. vitiosum* in the analyses. We believe that a more robust dataset would reflect better support for relationships, reveal new lineages and allow testing hypotheses of gene flow ans hybridization.

CONCLUSIONS

Our analyzes show the existence of a new specie lineage, closely related to *P. longisternalis* and identified intrapopulation lineages for both species. Furthermore, except for the lineage and *Pterinopelma* sp. All species identified a priori by morphometric analysis were recovered in molecular analyses. This reinforces the importance of a integrative method in phylogenetic studies. Still, despite being a preliminary study, given the limitation of our dataset, our result contributes to the hypothesis that morphological differentiation in Mygalomorphae species does not follow genetic diversification.

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CONCLUSÕES FINAIS

Os resultados morfométricos indicaram que as variações de estado morfológico dos principais caracteres diagnósticos entre machos e fêmeas de *P. longisternalis* e *P. roseus* são de caráter contínuo. Contudo, esse método foi capaz de recuperar ambas as espécies nas análises discriminantes. Além disso, a utilização dos valores médios da razão entre o comprimento e a largura do esterno é útil para discriminar as espécies.

Ainda, os resultados moleculares evidenciaram a existência de uma nova linhagem entre *P. longisternalis* e *P. roseus*. Cujo os caracteres morfológicos são crípticos aos de *P. longisternalis*. Contudo, foi decidido não realizar a descrição desta linhagem como uma nova espécie visto a falta de material disponível. Ainda, linhagens intrapopulacionais foram identificadas nas duas espécies analisadas, indicando que a diversidade do grupo é maior do que a vista por meio de caracterização morfológica.

Esses resultados reforçam a hipótese que os processos de diferenciação morfológica não acompanham a diferenciação genética em aranhas em Mygalomorphae e que análises integrativas compõe um método menos especulativo e mais preciso para estudar os processos de evolução do grupo.

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